food enzymes,
Carbohydrate metabolism,
polysaccharides,
Lipids, ~~fatty~~ acids,
Proteinases, Enzymatic
Browning,

Food Enzymes

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Food Enzymes

The first of a series of symposia on foods held at Oregon State College

Edited by

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Preface

In 1959, the State of Oregon celebrated its one-hundredth birthday. That same year was the fortieth year after Professor Ernest H. Wiegand first taught courses related to food processing at Oregon State College. It was also the year when the first **Symposium on Foods**—the first of an intended series—was held in the Department of Food and Dairy Technology at Oregon State College. To help commemorate the first two events, the third was arranged.

At the time that the first fish canning plant was put into operation on the Columbia River in 1866, the preserving of food, by any of the methods used then, was an application of *art* rather than of *science*. Not too much was actually known about the chemical components or characteristics of the foodstuffs which were to be preserved. Consequently, food preserving techniques had been improved more by trial and error than through a basic understanding of the problems which had to be solved. Professor Wiegand recognized this and formed and developed the Department of Food Technology during his thirty-three active years at Oregon State College on his belief that food technologists must understand basic scientific facts.

Furthermore, as time went on, the food industry became involved not only in preserving food so that it could be eaten at some future time, but also in changing its shape, texture, color, and flavor, by various chemical, physical, and biological means, and manufacturing into food *dishes* usually prepared only in the home. Although there is still the strong desire to preserve many of our foods in the natural state, most of the foods in the United States are sold to consumers in some other form.

The complexity of the food industry today and the dependence placed upon it by the population to maintain a high standard of nutrition, a high degree of convenience, and unquestionable safety, and to provide all of those qualities which result in the pleasures of eating, demand that more basic knowledge about foods be sought and used. In facing this need, it is immediately recognized that those persons who are engaged in basic research in branches of

chemistry and microbiology, for example, usually do not recognize the important implications of their research in respect to foods. On the other hand, the scientists and technologists who are doing research directly related to the food industry have not taken advantage of the knowledge possessed by the basic scientists.

To assist in building a bridge of understanding between the basic scientists and the applied scientists, the Department of Food and Dairy Technology at Oregon State College arranged the **Symposium on Foods: Enzymes,** which was held September 9 and 10, 1959. The subject of enzymes was selected because it was appreciated that many of the difficulties faced by the food industry involve or could involve enzymes, irrespective of commodity or method of preserving or of manufacturing. Because of their great capacity to influence both undesirable and desirable changes in foods, enzymes must be dealt with conclusively.

This book includes papers presented at the symposium by the participants who had been selected to give either the basic or the applied aspects of enzymes relating to carbohydrates, lipids, proteins, and oxidative browning. They are reviews of subject areas, for the most part, and should, it is hoped, stimulate a greater interest in food enzymes and bring the basic and applied scientists closer together in their efforts in the future.

The editor of this book has served only in the usual capacity of an editor and is indebted to the symposium participants for preparing their papers for publication, and to Dr. Ian J. Tinsley for his review of the manuscript. Editing Dr. Proctor's paper was saddened by the fact of his untimely passing just two weeks after he presented "Perspectives in Food Research."

Finally, but not least, we are deeply indebted to the National Institutes of Health for cosponsoring the symposium and providing financial assistance and encouragement.

H. W. Schultz

October 1, 1960

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Introduction to the Symposium

When I first entered the field of Food Technology enzymes were relatively unknown elements of nature which were considered in most cases as mere curiosities to food technologists. While they were recognized to cause some changes in foods, few technologists really realized the great importance of them to the food industries. As indicated above, they were considered unimportant structures of interest only to the "ivory tower" scientists.

A few years later I began to hear talk about oxidation and discoloration. In a few years the pendulum had swung so far that some were willing to attribute all types of discolorations to enzymes. As a matter of fact, this idea became so firmly entrenched in certain instances that it was necessary to expend a great deal of time and research effort to prove that such a thing as non-enzymatic discoloration could occur. Now we know it is a common phenomenon, a classical example of which is the so-called "browning reaction."

The sudden realization of the importance of enzymes in the food field developed a definite interest in these biological entities and we soon found them connected with pectin hydrolysis, rancidification, protein changes, and so forth. Even in these cases there were instances when the importance of enzymes was overestimated. For example, some assumed that the breakdown in meat was always enzymatic and couldn't result, for example, from a combination of moderately high temperatures and slight acid conditions which would favor breakdown in certain items. In other instances, it was assumed that all types of fat deterioration were caused by enzymes.

As time went on, some food technologists obtained a better understanding of enzymes and realized more and more their importance. Their point of view, however, was what one might term terminal. They were interested in what the enzymes did and how they might be controlled. Food technologists, however, gave little or no attention to the enzyme itself, its rate of reaction, character-

istics, isolation, and so forth. On the other hand, they were inclined to think of the Blotter Test for tomato products, the effect of hot break, cold break or activated hot break on the thickness of tomato products, and the effect of these treatments on the pectic enzymes. Here again the belief that pectic enzymes were the only factors responsible for the thickness or thinness of tomato paste was prevalent. Other factors such as particle size, presence of fibers, orientation of fibers, ionic strength causing clumping or spreading, solids, genetic factors, or cultural practices were overlooked. Of course, we now know that these factors as well as those involving enzymes are important in establishing the viscosity of tomato paste.

Food researchers eventually associated enzymes with changes in color other than those attributed to oxidase. There are certain instances when color components of foods are broken down, such as the decrease in concentration of anthocyanin pigments by anthocyanase. Information relating to some of these processes resulted in attempts at application. A classical example of this was the basic patent on blanching of green vegetables at 180°F., which is probably related to the maximum temperature (180°F.) for chlorophyllase activity.

Another example of the use of enzymes in control of color involves the use of pectinol treatment of crushed grapes for color extraction. Whereas some treatments may be for an increase in color, others are to maintain the color, and still others to decrease the color.

Texture and tenderness are also influenced by the enzyme activity. A classical example of the application and use of enzymes in this connection is the so-called Tenderay Process, whereby meats are hung at an elevated temperature in the presence of mold inhibiting light to enable faster and better aging. Natural enzymes in meats are more active at the higher temperature, the meat thereby becoming tender and edible faster. In line with this, attempts have been made to inject enzymes in hams and other meats and, of course, they are sold for tenderizing meats.

There has been some speculation about the possibility of using enzymes for tenderizing old and tough vegetables or for bringing about fiber breakdown. Some have wondered if old corn might not be converted into a more edible product by conversion of starch to sugar and perhaps bring about other desirable changes.

Only in recent years have the flavor aspects of foods been connected with enzymes. In many cases the development of flavors has been attributed to one enzyme activity or another. On the other hand, the use of enzymes for the production of flavor is relatively new. During the past few years considerable has been said about the use of flavorase for the improvement of flavor of cabbage and similar items. Whether or not the use of flavorase to improve flavor will find application remains to be seen. In any event, here is another potential application for enzymes.

Food technologists are using knowledge of enzymes to increase the stability of certain products. It is well known that starch in potatoes stored at relatively low temperatures is converted into sugar. On the other hand, sugar in potatoes stored at elevated temperatures is converted back to starch. These are enzymatic processes used in the potato dehydration industry to minimize the sugar content and thereby increase the storage stability of the dried products. Dried potatoes low in sugar undergo the browning reaction at a much lower rate than when the sugar content is high.

In another case the stability of dehydrated eggs can be increased by the direct application of glucose oxidation to the eggs. This removes the glucose and hence reduces the rate of deterioration by the so-called browning reaction.

During the past few years much has been said about the relation of the quality of raw materials to the yield and quality of processed product. It is more important than ever that we have a thorough understanding of the influence of cultural practices on the quality of a product and especially the relations of post-harvest physiological changes to the processing characteristics and quality of the final product. Respiratory and other enzymes may be extremely active during the post-harvest period. They can result in changes of flavor, sugar/acid ratio, texture, color, and so forth. In line with this, a thorough knowledge of enzymology by food technologists is most useful, although one time considered by some as useless information.

We now have a considerable number of commercial enzymes. These are finding more and more use in the food industries as time goes on. One of the oldest uses of enzymes is in the brewing industry. Amylases and proteolases inadvertently have been used in this industry for many years. In this industry one product, namely

malt, is produced essentially only for enzymes. The use of malt is very old and involves the early application of biochemistry. Truly, this was a remarkable discovery for a remarkable product. Only now are we starting to learn a little about what takes place and, because of the meager experience we have had at Davis, we are inclined to wonder how the old brew masters ever did as well as they did with such little knowledge about these complicated entities that do so much to produce the beverage that so many drink.

It is apparent from the examples given that we have arrived at a point where we now realize that enzymes are important in the food industries. We realize that they must be used and they must be controlled, but thus far we have been quite neglected in obtaining an understanding of the fundamental aspects of enzymes. We have relied on the biochemist to obtain information concerning mechanisms, kinetics, identification, characteristics, and so forth. Truly, the most fundamental work on enzymes is of interest to food technologists and belongs in a department of food technology just as much as in a department of biochemistry. Both groups should be working on enzymes, however, the point of view may be different. The biochemist seeks knowledge concerning the enzyme, whereas the food scientist working in the fundamental area of enzymology will always have in the back of his mind the use or control of these systems, yet the contribution of the two groups might be similar. By obtaining a thorough knowledge pertaining to the fundamentals of these reactions, we can do more in the way of preserving and producing better foods.

If we shoot at the stars or, as one of my colleagues puts it, "Smoke a little opium," we might speculate on the possibility of learning about groups of proteins that cause reactions. If we had such information as a result of fundamental studies perhaps we could construct enzymes that might do what we would want them to do. Why not build enzymes to break down pectin at 34°F. in one case or at 108°F. in another case? Why not selective tenderization of one item over another? Perhaps we could even visualize a case where we might have a mixed stew consisting of beef and lamb where the enzyme would selectively tenderize one type of meat and not the other.

Truly, the point of view of the food scientist has changed in certain centers and it must change in all. If we are to progress in

our treatment of foods, in our production of better foods, and the preservation of more foods, and if we are to create wealth by preservation, then we must get down to an understanding of the more fundamental aspects of enzymes. It is only the departments of food technology that look at these fundamental aspects that will make real progress in the future.

It is apparent that the food scientists at Oregon State College are thinking along these lines or they would not hold this conference. They are to be congratulated, and it is my sincere hope that other food scientists will follow suit.

David E. Green

The Future of Enzymology

The task of discussing the future of enzymology before a sophisticated audience is not an easy assignment. Peering into the future may be an amusing exercise in the privacy of one's laboratory, but a public performance is somewhat unnerving. My predictions cannot be documented. One can never really forsee some utterly unexpected development which overnight could change the whole course of scientific development like the discovery of nuclear fission or of the quantum behavior of the atom. Whatever is presented here is subject to these obvious limitations. It is doubted that you would want me to play so safe that I could at best be pedestrian and, by the same token, your patience would be stretched if discretion were thrown to the winds and my imagination ran high, wide and handsome. A middle course will be steered. It will be necessary for me to take a position, but the ideas presented will not be excessively wild—just mildly wild.

Upon my return to this country in 1940 after a sojourn of some eight years in Cambridge, England, the subject of enzymology was relatively unknown among American biochemists. There were, indeed, islands here and there where interest in enzymes was intense, but American biochemistry was still largly concerned with analytical procedures, with isolation and characterization of natural products, with metabolic studies on whole animals, and with nutrition. Since then there has been an explosive growth of enzymology. It was largely in the European laboratories that the foundations of the subject were laid in the period prior to 1940. But it was in this country during the 40's and 50's that enzymology skyrocketed from a pygmy to a giant dominating the field of biochemistry.

But now, after 20 years of triumph and almost phrenetic activity, enzymologists seem to have lost much of their fire and assurance. The classical problems have been pared to the bone, and many once-flourishing frontier areas have become quiescent. Is this the lull before the next storm or merely the aging of a meteoric discipline?

Enzymology has clearly spent its heroic youth and is now facing the less spectacular but more difficult challenges of maturity.

There are signs that we are in a transitional stage in enzymology and biochemistry, and therefore the time is appropriate to survey past accomplishments and to anticipate future directions and developments. It is important to bear in mind that the tremendous growth of enzymology would never have materialized so rapidly were it not for the fact that major advances in instrumentation and technology had made available powerful tools for exploiting the inherent advantages of enzymological studies. Equally important, there was an accumulation of 50 to 100 year's work on intermediary metabolism based on whole animal studies, and spectacular developments in genetic mutants which the enzymologist with his new and incisive tools could rapidly exploit. But enzymology cannot always be so fortunate, and in the years ahead the enzymologists will probably have to do most of their own groundbreaking, and that is a process which moves slowly.

First some of the high points in the development of classical enzymology will be reviewed briefly so that all can appreciate why progress was bound to be rapid at first, and then taper off as the probing pushed deeper and deeper.

A reaction catalyzed by an isolated enzyme has the great tactical advantage that only one overall chemical process is taking place without any complicating side reactions. This is an advantage which the organic and inorganic chemist rarely enjoys. It is not surprising that the enzymologist led the way in exploiting analytical tools such as the visible and ultra-violet spectrophotometer for monitoring chemical reactions. When A is converted to B it is almost always feasible to follow the reactions at a wave length which reflects either the disappearance of A or the appearance of B. The increase in sensitivity of the spectrophotometers made it possible to measure changes continuously at and below the μ molar level of concentration. One cannot exaggerate the value of being able to test the activity of an enzyme and get the answer in a matter of seconds. The easier it is to do an experiment, the more experiments are done and the greater the enthusiasm.

The tool of radioactive isotopes was on a par with the spectrophotometer in extending the range and sensitivity of enzymatic as-

says. Let me mention two striking examples. Fatty acid synthesis in cell-free extracts could be measured at first only by the incorporation of the label of radioactive acetate into long-chain fatty acids (Brady, Gurin, and Van Baalen 1952). It took five years before the enzyme system could be concentrated and defined to the point where net sythesis could be demonstrated by direct chemical methods (Wakil *et al.* 1957). The synthesis of DNA from deoxyribonucleotides in a cell-free system could be recognized only by measurements of radioactivity (Kornberg 1959). The amount synthesized involved only a few thousand molecules of nucleotides. Without this incredibly sensitive method of detection, the brilliant work of Kornberg and his colleagues would never have gotten off the ground.

The unique catalytic activity of an enzyme provides the most reliable guide to the purification of the protein with which the enzymatic activity is associated. The slightest alteration in the protein

TABLE 1

KNOWN COFACTORS OF ENZYMATIC PROCESSES

Hemes	Mg	Coenzyme A
Flavin	Biotin	Guanosine diphosphate
Thiamin	B_{12}	Cytidine diphosphocholine
Pyridine nucleotide	Tetrahydrofolic	Cytidine diphosphoethanolamine
Glutathione	Coenzyme Q	Uridine diphosphoglucose
Zn	Ascorbate	Uridine diphospho(sugar)
Mo	Adenosine monophosphate	Thioctic acid
Cu	Adenosine diphosphate	
Fe	Adenosine triphosphate	

can lead to the loss of activity. It was in the application to the purification of enzymes that the art of isolating pure proteins was finally perfected since the evaluation of isolation procedures could not be anything but reliable when judged by the supreme court of specific enzymatic activity. Besides, the ultracentrifuge and the electrophoresis apparatus simplified enormously the task of defining with precision the degree of purity of isolated enzyme proteins.

Hundreds of enzymes have been isolated and documented and many of them crystallized. Any beginning graduate student can now carry off the isolation of a new enzyme with a reasonable assurance of success if his technique and knowledge of the literature is good. Whether the enzyme is present in traces or in substantial amounts the means are readily available for isolation and purification.

The ease with which enzymes can be isolated had as an important

consequence a speed-up in the recognition and discovery of the cofactors and functional groups of enzymes. Table 1 contains a list of the authentic cofactors and functional groups which have been uncovered to date. In our laboratory within the past two years the catalytic role of biotin in the carboxylation of acetyl CoA to malonyl CoA has been established (Wakil *et al.* 1958), and a new coenzyme of the electron transport sequence of mitochondria has been discovered, vis., coenzyme Q (Crane *et al.* 1957).

TABLE 2

DEGRADATIVE SEQUENCES DEFINED AT THE ENZYMATIC LEVEL

Fermentation	Hydrolysis of polysaccharides, lipides, proteins, and nucleic acid
Glycolysis	Purine and pyrimidine breakdown
Citric Cycle	Glyoxylic cycle
Pentose Cycle	Oxidation of amino acids and derivative acids
Urea Cycle	
Fatty Acid Oxidation	Oxidation of C_1 compounds

TABLE 3

SYNTHETIC SEQUENCES AND PROCESSES DEFINED AT THE ENZYMATIC LEVEL

Synthesis of polysaccharides	Synthesis of nucleotides
Synthesis of hexoses and disaccharides	Synthesis of purines and pyrimidines
Synthesis of lipides (simple and complex)	Synthesis of amino acids
Synthesis of RNA and DNA	Methylation
Synthesis of heme	Thiolation
Acetylation	Hydrogenation
Sulfatation	Acylation
Phosphorylation	
Amination	

The enzymological approach to the problem of the definition of the individual steps in a complex sequential process has such obvious advantages that it is not surprising that practically every such process which survives the disintegration of the cell and which involves soluble enzymes has been brought to heel. In Tables 2 and 3 have been listed degradative and synthetic processes which have been defined in detail at the enzymatic level, and these lists encompass the major metabolic events of living cells.

The strategy of divide and conquer has been the key to the enzymological approach. Take any one enzyme in the complex process; define the reaction catalyzed and the product formed; and then it is only a matter of time, patience, and skill before the entire picture unfolds.

The study of the individual enzyme simplifies enormously the task of both isolating and characterizing the product of the reaction. One does not have to be an accomplished chemist to isolate a product which is largely pure to begin with and whose chemical composition is in large measure known beforehand. To make matters easier the most incredibly efficient devices for purifying organic compounds in large or small amounts have been at the beck and call of the enzymologists during the past two decades. Paper and column chromatography combined with techniques such as the use of radioactive isotopes have made the isolation and characterization of metabolic intermediates a relatively straightforward task.

The enzymologist has not only borrowed techniques but has also contributed techniques which have found wide applications in other disciplines. The most ingenious of these techniques has been the use of enzymes as reagents for identifying parts of organic molecules and for defining the exact position of certain groups in the molecule (Racker 1956). A large number of highly selective enzymes has been assembled which makes it possible to establish the structure of an unknown nucleotide or carbohydrate or peptide available only in μgram amounts in a matter of a few days. The use of enzymes of restricted specificity for the stepwise degration of proteins or carbohydrates or lipids is commonplace today. The power of the enzymological approach to the elucidation of structure cannot be overestimated.

The rapid development of enzymology had as one of its major consequences the liquidation of some of the classical problems of structure—the structure of ribose and deoxyribose nucleic acid, the structure of complex lipids and of polysaccharides, and the structure of nucleotides. It is only the proteins which have been more than a match for these new tools. Even in that area some of the low molecular weight proteins and polypeptides have already succumbed to the new tactics of the enzymologist and the biochemist (Sanger 1956; du Vigneaud *et al.* 1953).

The concept of ATP as the driving force in biosynthesis and of the conversion of oxidative energy to the bond energy of ATP is undoubtedly one of the most significant achievements of the enzymologist and one of the great unifying principles of biology (Lipmann 1946). On the one hand there are the primary oxidative systems such as glycolysis or the citric cycle which generate ATP, and on the

other hand there are the large number of synthetic processes which have to be initiated or triggered by ATP (summarized in part in Table 3). We know a great deal about the ATP-requiring reactions but less about the ATP-generating systems, except for the generation of ATP in glycolysis (Warburg and Christian 1939). When we come to discuss the problems of the future, the mechanism of oxidative phosphorylation and photosynthetic phosphorylation will certainly have to be included among the major unsolved problems.

To complete this running commentary on the high points of enzymological achievement, a few words about the properties of enzymes should be said. Much progress has been made in the understanding of the *modus operandi* of enzymes. The enzyme-substrate complex has at last been established by direct chemical evidence as in the case of the fatty acyl dehydrogenases (Steyn-Parvé and Beinert 1958). The reality of binding sites and even the nature of the binding groups in the protein moiety of the enzyme have been established in a few favorable cases. A theory of enzyme kinetics based on first principles and of acceptable rigor is beginning to be developed (Alberty 1956). The nature and properties of the active groups of enzymes are being intensively investigated, and much of the mystery which enshrouded this problem is fast being dissipated.

In retrospect it would appear that a whole series of nicely timed instrumental and analytical developments in other fields made it possible to clean up the problems of enzymology of a classical nature in record time. Where it was merely a question of isolating an enzyme or a cofactor, or a product of reaction of a system of multiple soluble components, the problem was no match for the methods. The wall was reached when the problems under attack could not be resolved in such simple terms. When enzymologists had to grapple with the mechanisms of hormone action, or oxidative phosphorylation, of gene action, or muscular contraction, or protein synthesis, to take a few choice examples, the pace became painfully slow, and the poverty of tactics and strategy painfully obvious.

There is yet another commentary on past developments which has to be made in order to put things in perspective. Enzymology has up to now almost completely by-passed the problem of biological organization, and this was achieved by concentrating on those aspects of the problem which could be solved without reference to organization. It is possible to learn how glucose goes to lactic acid

without knowing anything about the disposition of glycolytic enzymes in the cell. The details of the citric acid cycle can be worked out without even knowing of the existence of the mitochondrion. In fact, the emphasis on the facets of enzymology, which can arbitrarily be divorced from considerations of biological organization, convinced a large number of enzymologists and biochemists that biological organization is largely an invention of imaginative microscopists and anatomists. There are even today some biochemists who believe that any biological process can be reconstructed by putting together each of a large number of isolated enzymes and coenzymes in soluble form, and presto, the muscle will contract, the brain will send signals, and the kidney tubule will secrete glucose. There has been no basis or encouragement for this indifference to the biological realities. Wherever enzymologists have locked horns with problems which have an unmistakable organizational component they have found no way of getting around organization, and they have found classical methods utterly inadequate to solve these problems. Classical enzymology has made little headway in dealing with the problems of the mitochondrion, the chloroplast, the muscle system, the cell membrane, the microsomal nucleoprotein fraction, and the granules of the endoplasmic reticulum.

In the enzymology of the future the intimate relation between the structure and function of biological catalysts will be the actual theme, and this in turn will be the link between enzymology and the other biological disciplines.

The structure of the mitochondrion determines the function of the mitochondrion, and if the passage or transfer of electrons within the mitochondrial matrix leading to the esterification of inorganic phosphate is to be understood, it will be necessary to know in detail how all the elements of the electron transfer chain are arranged one with respect to the other and how this arrangement makes possible the coupling phenomenon. This concept of enzymatic function as an aspect of biological organization underlies some of the most basic events of living cells. The biochemical machines which convert energy from one form to another, the genetic apparatus, the protein-synthesizing systems, the instruments of organ function and the mitotic apparatus are all extensions and expressions of this fundamental structure-function relationship.

In the new enzymology it will be important to know not only what enzyme A does, but how enzyme A is linked to other components in a particle, and to which components, and how this arrangement of A with respect to the other components facilitates the function of the particle. Position and arrangement of parts assume equal importance in the new enzymology with the function of the individual enzyme, and the function of a unit of several or many enzymes becomes as fundamental an objective as the function of a single enzyme.

If the fact of organization can be accepted and looked upon as a chemical problem no different in kind from any other chemical problem, then there is no doubt that, in the not too distant future, how a mitochondrion works will be understood in as much detail as the sequence of events in fatty acid oxidation is understood. Once the enzymologist has found the keys to the understanding of the structured elements of the cell then there will be few cellular events which can elude biochemical inquiry.

Enzymologists among the food technologists have long been aware of the importance of structural considerations in the preservation of biological products and particularly fruit. When structure is damaged, as in the bruising of fruits, the rapid enzymatic events which ensue are tokens of the subtle manner in which enzymes are compartmentalized and rendered inaccessible to their appropriate substrates. The problem of preventing the structural changes and the enzymatic holocaust which freezing induces is not a new one to the food technologist. Some of the key problems of food technology center around the subtle connection between the state of tissues and their enzymatic activities.

Biochemistry has had minimal success in elucidating the mechanism by which hormones control and regulate cellular events and organ function. It is possible that in one or two cases hormones may exert their effects directly on specific enzymes (Talalay and Williams-Ashman 1958), but the signs are multiplying that the targets of hormones are structured and organized systems such as the cell membrane (Levine and Goldstein 1952). The preliminary to the solution of the mode of action of hormones is surely the isolation and characterization of the membrane systems in biochemical and enzymological terms.

Just reflect for a moment on the long list of therapeutic agents which are the stock-in-trade of the medical profession—aspirin, digitalis, codeine, morphine, barbiturates, tranquillizers, anaesthetics, adrenaline, eserine—to mention the first few that come to mind. Nobody has yet the foggiest idea how any of these act. This state of ignorance is not the result of any lack of interest on the part of enzymologists. There have been innumerable efforts to relate the mode of action of these agents to effects on specific enzymes, but in all cases a blank has been drawn. The conclusion is obvious that the isolated enzymes of classical enzymology are rarely, if ever, the targets of therapeutic agents. When these enzymes are studied out of the context of the structured elements of which they are integral parts in the cell, it is impossible to come to grips with the mechanism of action of these pharmacological and therapeutic agents. The most fascinating problems of biology—the mode of action of most pharmacological and therapeutic agents, the mechanism of brain, kidney and muscle function, the operation of the genetic apparatus, the homeostatic controls of the body, the conversion of energy from one form to another, the regulation of cell growth and differentiation —all lead to the same gap in our knowledge—the understanding of the structured elements of the cell.

The next revolution in enzymology will undoubtedly coincide with a breakthrough in grasping the operational principles of biochemical machines and complex systems. When it is possible to spell out the chemical strategy by which a protein is built up from component amino acids, how templates for protein synthesis are arranged and constructed, how hormones modify cell membranes, how cell membranes regulate the flow of solutes, how an electrical impulse is generated in a nerve cell, how a mitochondrion couples oxidation to phosphorylation, how the chloroplast couples photochemical cleavage of water to phosphorylation, then a new and fantastic era of exploration and systematization will be our reward. We could then think in terms of a frontal assault on the classical problems of medicine, those of disease and senescence. Just as the virus has acquired the capacity to control and direct the synthetic mechanisms of the host cell, so in time the enzymologist may look forward to a comparable achievement—that is the regulation of cellular processes for the betterment of mankind.

These predictions may at present seem visionary, but I venture to

suggest that in the next 25 years many of the objectives to which I have referred will be approached, if not attained. If we set our research sights high and give our young and imaginative enzymologists the tools and encouragement to grapple with these formidable problems there is no question of our ultimate success.

Any one of the many problems of the future that has been touched upon are far beyond the capacity of a lone investigator, however gifted, to tackle singlehanded, even in a lifetime. This is a new and uncomfortable thought for most biochemists, but we shall have to get used to it just as the physicist was obliged to learn to live with this inescapable reality. There are problems which by their very nature are so complex or which have so many facets that it is foolhardy for any one individual to grapple with them. Practically all the major problems of biochemistry and enzymology now fall into that category.

Research in our universities will have to assume a cooperative or team basis if the universities are to continue to play their part in the development of enzymology. The only other alternative is to work on problems which are no longer primary in the development of the science.

The dividing line between the subject matter of enzymology and that of other biological disciplines is now rapidly disappearing. Consider some aspects of the virus problem. (1) The virus has been shown in several instances to contain an enzyme capable of hydrolyzing the mucopolysaccharides of the cell wall of the host cell (Burnet 1951). (2) The apparatus in the tail of the virus which facilitates the penetration into the cell of the nucleic acid core involves, according to Kozloff and Linte (1959), a contractile protein not unlike myosin and a supply of ATP for triggering the contraction. (3) The viral nucleic acid core which penetrates the host cell induces the formation of enzymes which are required for the synthesis of viral RNA and suppresses some of the normal enzymatic functions of the host cell (Hershey 1956). (4) The virus makes use of the existing enzymatic machinery of the host cell to produce all the component parts which are essential for synthesis of more virus—this includes proteins, nucleic acid, and ATP. If looked at properly and realistically, an important sector of virology is a branch of enzymology. Enzymology and pharmacology, enzymology and physiology, enzymology and microbiology, enzymology and genetics—these are just

a few of the foreordained marriages of the near future. The practical implication of this merging of various fields is that the enzymologist of the future may be well advised to be a competent and well-trained biologist if he is to play a role in the significant new developments in these hybrid areas.

The science of genetics has exerted an enormous influence on enzymology and in some areas genetics and enzymology have fused. That genes ultimately determine which enzymes will be formed is current dogma, but no one knows in detail the causal links between genes and enzymes. We shall have to know a great deal more about the mechanism of protein synthesis, about the relationship of RNA to protein synthesis and of genes to DNA and RNA synthesis before the enzyme-gene hypothesis can acquire biochemical precision.

It is now well established that the synthesis of certain enzymes in bacteria and even in animal tissues can be induced by addition of the appropriate substrate (Spiegelman and Campbell 1956; Knox 1958). Is this perhaps a more general phenomenon which is not easy to recognize since only rarely is the appropriate substrate lacking? The converse phenomenon, the deletion of enzymes, is of great current interest to those concerned with the etiology of cancer (Rush 1956). Carcinogenic agents in many cases lead to the deletion of certain enzymes which in turn modify the metabolic pattern of the affected cell, and this carcinogen-induced alteration is believed to be one of the primary factors in the formation of a cancer cell from a normal cell. The knowledge of the factors which control enzyme formation may provide the enzymologist with one of the most effective tools for combatting disease and senescence.

A mitochondrion is built up of at least 30 large protein molecules not to mention lipid. These structural components form a very precise and reproducible molecular unit. How is this accomplished? Are all the parts put together under enzymatic control in a seriatim fashion or is each part so designed that it can only fit the appropriate partner, and so the mitochondrion can be assembled merely by mixing all the parts in solution?

It can be seen that there is a vast area of ignorance of the how and when of enzyme synthesis. At what stage in embryonic development are enzymes laid down? Does the mitochondrion suddenly appear or does it get built up section by section during a measurable

time interval? Genetics, physiology, embryology, and enzymology will all have to team up to deal with these fundamental problems.

Just as it is inevitable that enzymology will assume a more biological orientation, it is equally probable that theoretical chemists will become increasingly more interested in enzymology. Enzyme systems offer unique opportunities to the chemist for studying classical chemical problems such as free radical formation during oxidoreductions of flavin enzymes, reversible denaturation of proteins with enzymatic activity, the relation between amino acid sequence and catalytic potentiality, the molecular basis of enzymatic function *et cetera.* Enzyme kinetics are being studied with great skill by theoretical chemists, and this area is gradually being dominated by the professional chemist. The molecular basis of enzymatic catalysis has attracted theoretical organic chemists who are now in the forefront of efforts to describe enzymatic processes mechanistically. The photosynthetic process has been a lodestone for a host of physical and photochemists. In fact there is hardly a department of chemistry in the country which does not count at least one member who is concerned with one or another aspect or enzymology.

The choice for the enzymologist of the future is either a strong biological or a strong chemical orientation. One will have to achieve a high degree of competence and sophistication in one or the other discipline to be equal to the research tasks ahead.

Although a consideration of this kind cannot help but be too general and too sweeping, nonetheless it is always of value to look ahead to the future. The rate of obsolescence and fossilization in science is a very rapid one. The exciting problems of today are overnight the dry bones of tomorrow. It behooves all of us to think occasionally of the overall status of our science lest we, too late, find ourselves in the backwater.

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Vernon H. Cheldelin

The Metabolism of Carbohydrates

The following two Chapters are to be devoted to enzymes affecting carbohydrates. This important class of materials may, for convenience, be discussed in two ways: First, as they exist in their macromolecular structure (starches, gums, cellulose, pectic substances, etc.); and second, as they provide energy for living. The present paper will emphasize the latter role.

All organisms on this planet must ultimately obtain their energy from sunlight. However, most of us (except green plants and a few photosynthetic microbes) receive our energy in some secondary form, as stored chemical energy. This stored energy is found to the greatest degree in nature, as carbohydrates. The carbohydrates are thus of major importance to all students of life processes, and especially to food technologists.

The story of the conversion (metabolism) of carbohydrates to water, carbon dioxide, and energy is extremely complex. Full knowledge of the intricate changes involved has become available only during relatively recent years; indeed, the story as we know it today may yet be incomplete in certain respects. The details are sufficiently well known, however, to permit the charting of at least some major trails through the forest of chemical reactions that characterize carbohydrate metabolism in the living cell, and it is these trails that I wish to explore with you today.

THE TRICARBOXYLIC ACID CYCLE

A discussion of carbohydrate metabolism should take notice at the outset of one of the major landmarks not only in this field, but in all biochemistry—the Krebs tricarboxylic acid cycle. It is now 23 years since Krebs and Johnson (1937) published their classic paper which showed that pyruvic acid, as it is produced by degradation of sugar (glucose) can in turn undergo a series of reactions to be burned completely to carbon dioxide and water. Although many of us may not have seen the structure of the intermediate products recently, the essential completeness of the process can be captured by con-

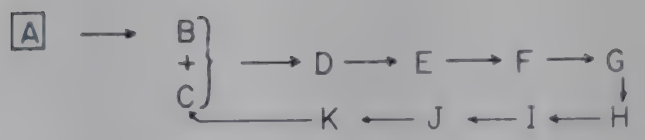


FIG. 1. THE KREBS TRICARBOXYLIC ACID CYCLE

A schematic diagram

sidering the steps in a wordless sequence, as in Fig. 1. In this simplified diagram, compound *A* (pyruvate) breaks down to give a derivative of acetic acid (*B*). This condenses with another acid, oxalacetic (*C*) to give citrate (*D*). Hence the name citric acid cycle, by which the whole cycle is sometimes called. The compounds that follow the breakdown of citrate are in succession, *cis*-aconitate, isocitrate, oxalsuccinate, α-ketoglutarate, succinate, fumarate, and malate (*K*). All this is of course important to the chemical biologist, but the *really* important fact at this point is that malate (*K*) in its turn breaks down to regenerate oxalacetate, which is compound *C*. Thus, the cycle can start over again, using a fresh molecule of compound *A*. This compound (pyruvate) which comes from sugar is cycled to carbon dioxide and oxygen, with one of the intermediates (*C*) re-emerging at each turn of the cycle and accepting a fresh molecule of *B* until all of the sugar is used. The cycle in its more conventional form is given in Fig. 2.

The elegance of the foregoing scheme caught the interest and imagination of biochemists of the late 1930's on a world-wide basis. Within a few years investigators in many countries had examined various animals and plants, and had discovered Krebs cycle activity to be present and apparently operative. Indeed, as new organisms were tested, it appeared that, except for a few micro-organisms, there were very few species that did not contain the Krebs cycle complex.

THE MITOCHONDRION AND THE CITRIC ACID CYCLE

The ubiquity of the Krebs citric acid cycle was given a new dimension of appreciation in the early 1940's when Krebs cycle activity was discovered in the mitochondria, or "large granules" of the cell.

Let us review briefly the recognizable parts, or inclusions, that occur within a living cell. The diagram in Fig. 3 represents a cross

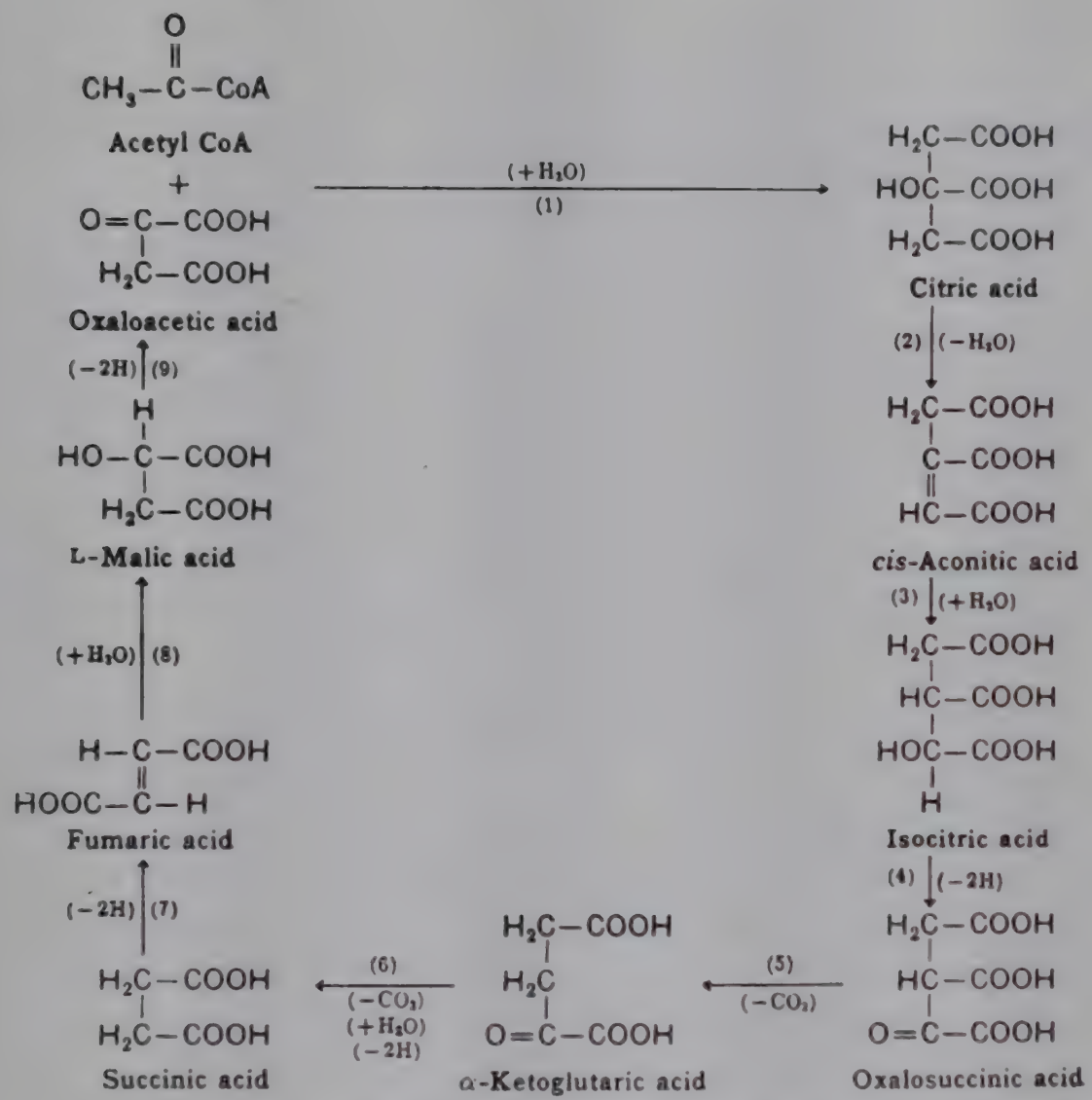


FIG. 2. THE KREBS TRICARBOXYLIC ACID CYCLE

section of a generalized cell, with the large nucleus near the top of the figure. The section that is sketched from the nucleus to the outer edge of the cell contains numerous relatively large inclusions, called the mitochondria, as well as many other smaller particles. The finer lines that appear, as well as some of the solid dots, represent the endoplasmic reticulum, or microsomes.

These subcellular particles have been recognized by cytologists for many years. The mitochondria are clearly visible under a high-power microscope, and the microsomes may be recognized with ease in electron micrographs, such as the ones provided by Palade (1955, 1956) and shown in Fig. 4, 5, and 6. More importantly, beginning about 1940 Claude (1941, 1946) and others observed that the oxidative capacity of the cell (leading to energy formation) appeared to reside within the mitochondrion. The data in Table 4

TABLE 4

KREBS CYCLE OXIDATIONS IN RAT LIVER FRACTIONS[1]

The fractions were tested at equal levels of total nitrogen. The system contained 0.01M substrate, 0.005M $MgCl_2$, 0.05M KCl, 0.001M ATP, 0.01M phosphate buffer, and 10^{-5}M cytochrome-c.

Substrate	Mitochondria μL	Nuclei μL	Microsomes Plus Soluble μL
Citrate	180	11	8
α-Ketoglutarate	184	21	8
Pyruvate + oxalacetate	199	28	8
Succinate	244	33	14
Malate	161	8	6
None	14	8	6

[1] After Lehninger (1951).

TABLE 5

COMPLETE OXIDATION OF KREBS CYCLE INTERMEDIATES BY RABBIT KIDNEY[1]

The complete system in every case contained 1.5 ml. of residue R_3K, 0.3 ml. of 0.01M adenosine triphosphate, 0.2 ml. of 0.02M magnesium sulfate, 0.2 ml. of 0.125M phosphate buffer of pH 7.2, and substrate in the amount indicated. In the main compartment of both the experimental and the blank manometer vessels, 1 μ mole of succinate was added to prevent the deterioration of the enzyme during the equilibrium period. After five minutes, the taps were closed and the substrate solution in the side arm was then mixed with the cup contents.

		Oxygen	
Substrate	Substrate Added μMoles	Observed μAtoms	Theory for Complete Oxidation μAtoms
α-Ketoglutarate	5	38.1	40.0
Malate	5	30.7	30.0
Malate	10	56.8	60.6
Citrate	5	47.1	45.0
Isocitrate	5	45.3	45.0
Succinate	5	34.3	35.0
Fumarate	5	34.0	30.0
Pyruvate	5	23.9	25.0
cis-Aconitate	5	48.4	45.0
Oxalacetate	2.5	12.2	12.5
	Substrate Oxidized μMoles	Carbon dioxide μMoles	
α-Ketoglutarate	4.37	21.3	21.9
Isocitrate	3.79	21.7	22.9
Succinate	3.77	15.1	17.2
Fumarate	4.73	16.4	18.9
cis-Aconitate	5.00	27.9	30.0

[1] After Green (1951).

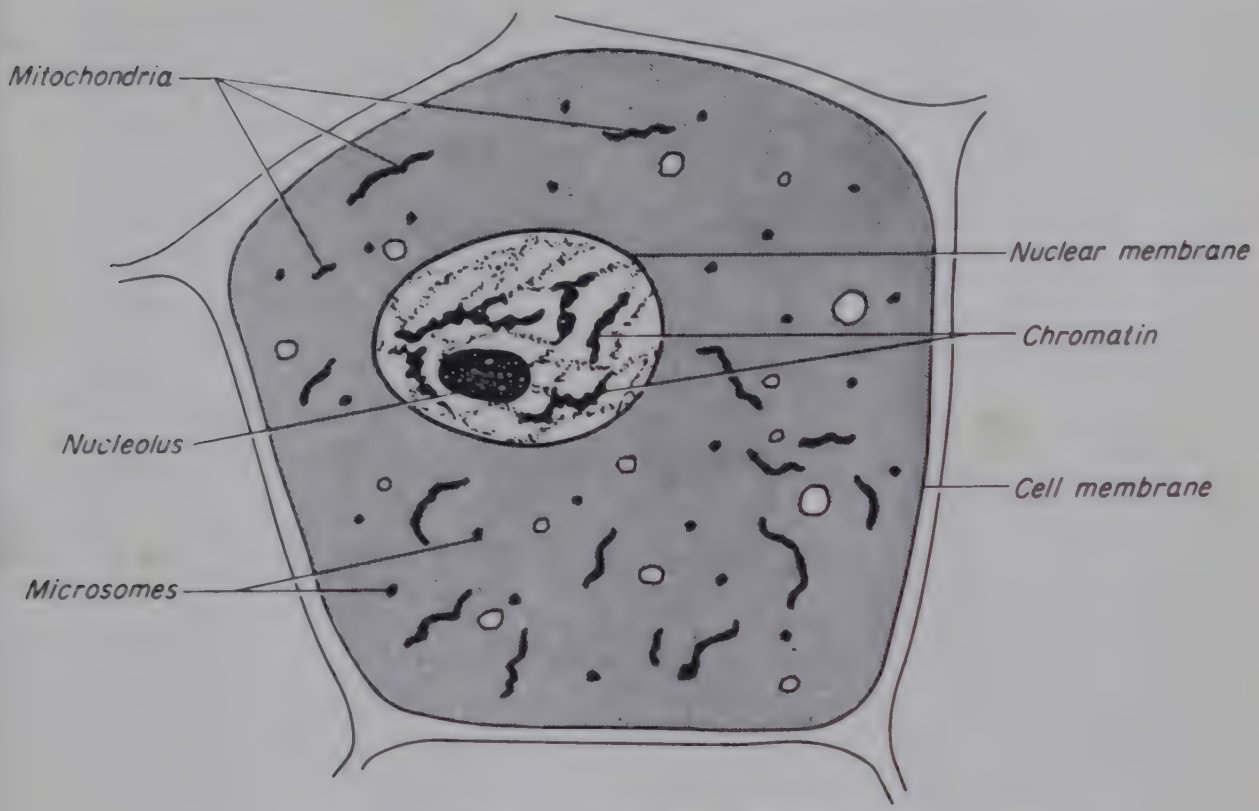


FIG. 3. CROSS-SECTION OF A GENERALIZED CELL

are indicative of the comparative oxidation capacities of the various organelles within the cell with respect to the Krebs cycle intermediate. Thus, only the mitochondria exhibit the ability to oxidize these substrates; the nuclei (which contain our genetic reduplication coding material) and the microsomes (which are involved in protein synthesis) are quite unable to promote Krebs cycle oxidations.

Table 5 is presented to show the *complete* oxidation of citric acid cycle members by mitochondria. It is clear from an inspection of the data that each of the members of the Krebs cycle tested is converted to the theoretical amount of carbon dioxide, with theoretical consumption of oxygen in the process. It is also clear from Tables 4 and 5 that the mitochondria are able to oxidize completely the first great class of foodstuffs, i.e., carbohydrates, and that this constitutes a "division of labor" within the cell, since the nuclei and microsomes do not participate in this activity.

OTHER OXIDATIONS BY MITOCHONDRIA

Although it may seem at first superfluous to the story of carbohydrate breakdown, Table 6 and Figs. 7 and 8 are added here to

TABLE 6

OXIDATION OF CAPROIC ACID TO ACETOACETIC ACID, CARBON DIOXIDE, AND WATER IN RABBIT LIVER[1]

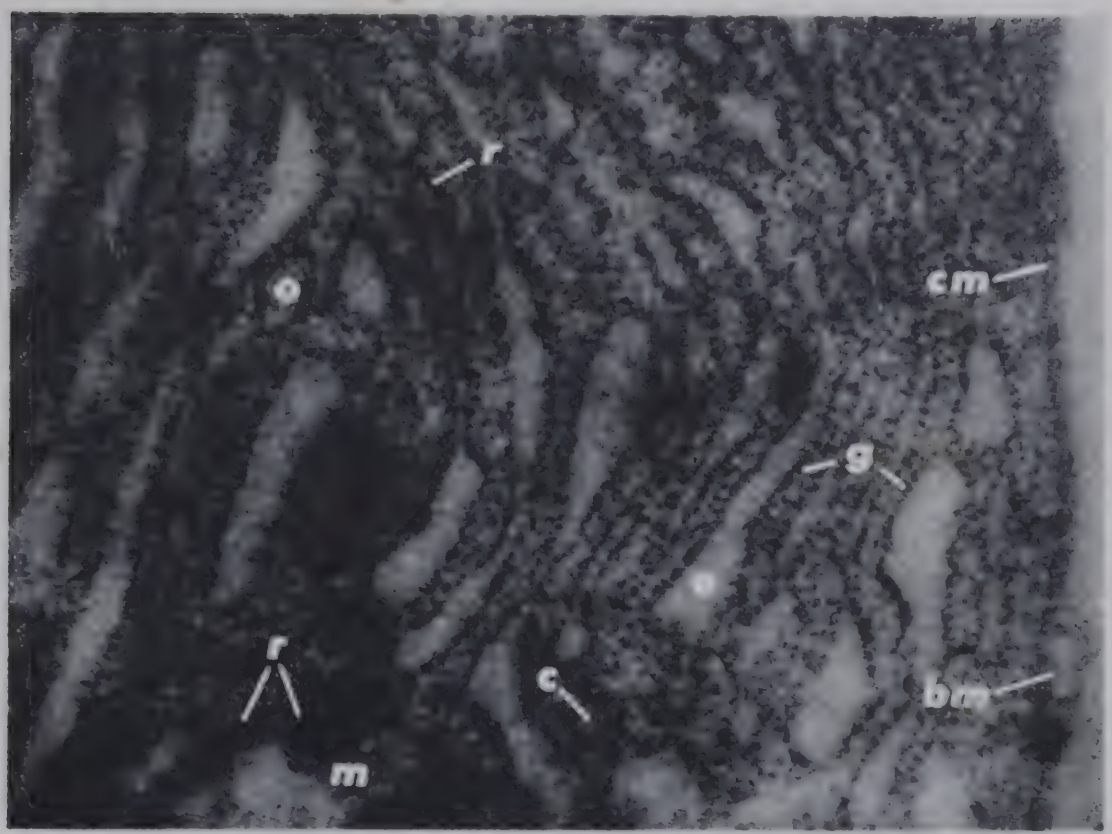
Caproate	Theoretical Oxygen Requirement	Oxygen Consumed	AcAcOH Produced	Caproate Oxidized, Calculated from Oxygen Consumed	
μMoles	μAtoms	μAtoms	μMoles	μMoles	O_2/CO_2
15	120	105	13.2	13.1	3.97
15	120	101	13.1	12.7	3.85
15	120	96	13.1	12.0	3.68
30	240	92	12.1	11.5	3.80
30	240	142	17.9	17.8	3.93
30	240	163	21.9	20.4	3.74

[1] After Cheldelin *et al.*, 1950.

further emphasize the importance of the mitochondrion in oxidizing the other major classes of foodstuffs. As we shall see, the oxidative pathways are all closely related. Let us first consider the fats.

Fats and Fatty Acids

Through an exacting series of investigations by many scientists, beginning with Knoop in 1904 and culminating in the efforts of Green and coworkers in 1953, (see Beinert *et al.* 1953), it has been established that the long chain fatty acids that occur in natural fats, are oxidized in the body by means of the mitochondria fraction in the individual cells. When fatty acids are converted to energy in the kidney or striated muscle, for example, the long carbon chains are "scissored" off, two units at a time, according to the scheme given in Fig. 7. Thus, the eight-carbon fatty acid, octanoic (caprylic) acid, is shorn of two carbon atoms *which are the same two carbons as compound B in Fig. 1.* This compound, acetyl coenzyme A, then goes directly into the Krebs cycle where it is oxidized to carbon dioxide and water just as if it had arisen from carbohydrate. The remaining portion of the fatty acid, which is the six-carbon hexanoic (caproic) acid, undergoes the same "scissoring" treatment, producing another unit of acetyl coenzyme *A;* and so on to the end. Because the remaining fatty acid unit is smaller at each turn than the preceding one, Lynen and Ochoa (1953) have referred to this process as the fatty acid helix rather than a true cycle—even though the process is generally cyclic in character. The important feature for the present discussion is that the enzymes that are responsible for



After Palade (1955)

FIG. 4. MITOCHONDRIA AND MICROSOMES IN THE PANCREAS

Electron micrograph of a limited field in the basal region of an acinar cell of the pancreas (rat). The cell membrane (*cm*) is coated towards the exterior by a poorly defined layer of dense material (*bm*) that may be the equivalent of a basement membrane. Part of a mitochondrial profile appears at *m*.

The rest of the field is taken up by elongated (*e*), oval (*o*), and circular (*c*) profiles of the endoplasmic reticulum which, in this case, exhibits a certain degree of orientation as shown by the general parallelism of the profiles to the cell membrane. The cytoplasmic matrix, slightly denser than the homogeneous content of the endoplasmic reticulum, is disposed in bands in between the profiles.

Note that in the matrix there are numerous small and dense granules (*g*) which appear to have particular affinity for the membrane limiting the cavities of the endoplasmic reticulum. The outside surface of this membrane is actually covered by many such particles which in a few places (*r*) appear to be more or less regularly disposed in rows.

Note also that the granules do not show any affinity for the cell membrane (inner surface) and for the membrane limiting the mitochondrion. ×50,000.

promoting these oxidations of fatty acids, reside predominantly in the mitochondria of the cells; and thus we can say that the consumption of fats, which constitute the second great class of foodstuffs is also (exclusively) a property of the mitochondria. This is demonstrated in Table 6; the fatty acid taken for study here is caproic, the six carbon member, showing its consumption by liver mitochondria. Liver mitochondria form acetoacetic acid as a by-product in fatty acid oxidation, at a rate of one mole per mole of fatty acid. This ratio

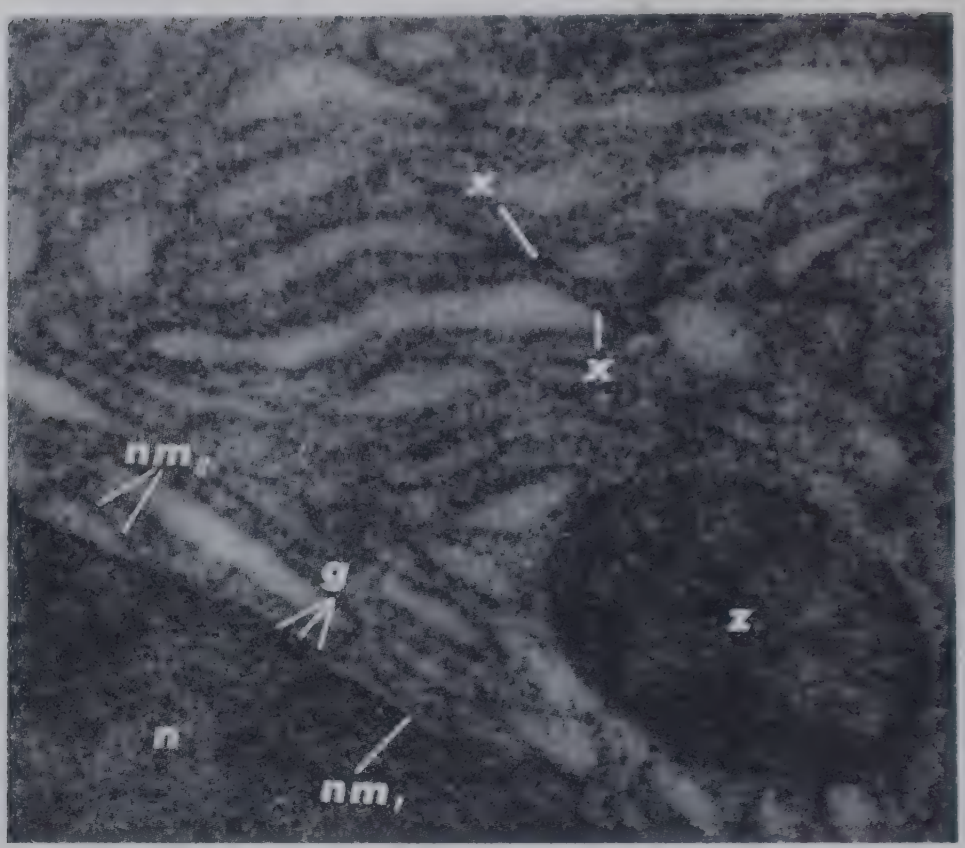


FIG. 5. MITOCHONDRIA AND MICROSOMES IN THE PANCREAS

Electron micrograph of a limited field in the vicinity of the nucleus (n) in the same cell as in Fig. 4. The profiles of the endoplasmic reticulum are equally diversified in shape and in this field appear to be predominantly oriented around the nucleus. Small, dense particles are present in the cytoplasmic matrix.

At x the membranes limiting two adjacent profiles have been normally sectioned as indicated by their extreme thinness, high density, and sharp outline. Such sections show to advantage the relationship between the membrane and the small granules: the latter appear in close contact with, as if "attached" to, the outside surface of the membrane.

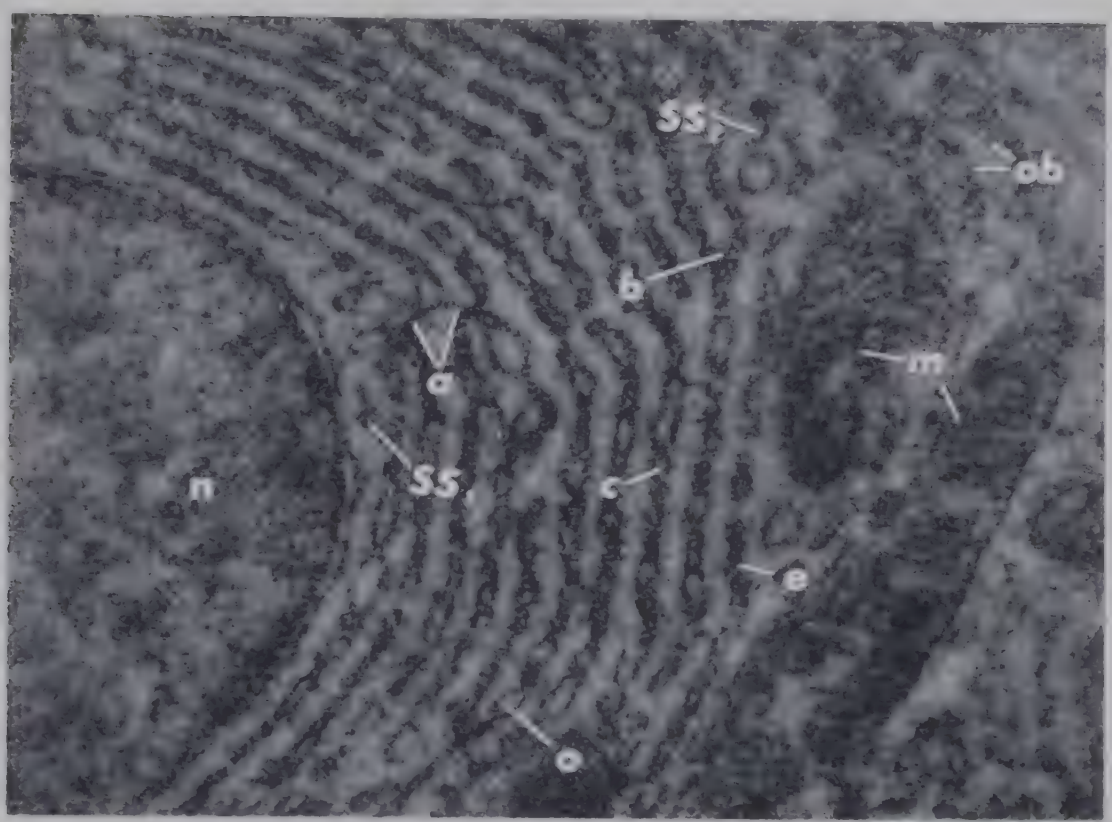
Note that the membrane limiting the cytoplasm towards the nucleus (nm_2) is not rigorously parallel to the nuclear membrane proper (nm_1), and that the former is dotted with small granules (g) like the membrane of the endoplasmic reticulum.

The large dense body marked z is a zymogen granule. ×50,000.

is used in Table 6 to check the agreement between observation and theory in caproic acid disappearance, oxygen consumption, and formation of oxidation products.

Proteins

As with the fatty acid oxidation, the oxidation of amino acids, which constitute the building blocks of all proteins, is predominantly a property of the mitochondria. Although no individual data are presented here, this observation has been made by several workers;



After Palade (1956)

FIG. 6. THE ENDOPLASMIC RETICULUM

Pancreatic exocrine cell (guinea pig).

The figure shows a relatively large field in the basal region of an acinar cell. The nucleus appears at *n* and two mitochondrial profiles at *m*. The rest of the field is occupied by numerous profiles belonging to the endoplasmic reticulum (*ER*). Almost all of them are of a rough surfaced variety and appear aligned in rows which are disposed parallel to one another at more or less regular intervals. In this case the entire arrangement is concentric with the nucleus. Note that this orderly disposition is disturbed in a few places by branching rows (*b*) and by anastomoses (*a*) between adjacent rows.

Note that although elongated profiles (*e*) predominate, circular (*c*) and oval (*o*) profiles are also present in many rows. In three dimensions these rows correspond to fenestrated cisternae or to reticular sheets.

In the middle region of the figure, the *ER* elements are normally sectioned and show clearly their lumen, limiting membrane, and attached granules. In the upper left corner similar elements appear in oblique section (*ob*).

Smooth surfaced elements (SS_1, SS_2) are very rare in pancreatic acinar cells. When present they frequently occur in small clusters (SS_1). ×20,000.

it is summarized in the diagram in Fig. 8, where one sees that the various amino acid oxidizing enzymes form products which again are components of the Krebs cycle (compounds *A*, *H*, and *I*, *J*, *K*, or *C* of Fig. 1). Thus, alanine, valine, or serine enter the Krebs cycle through the "C_3" or three-carbon gateway; glutamic acid and several others gain entry through α-ketoglutarate, the "C_5" compound in the Krebs cycle; whereas threonine and aspartic acid disappear

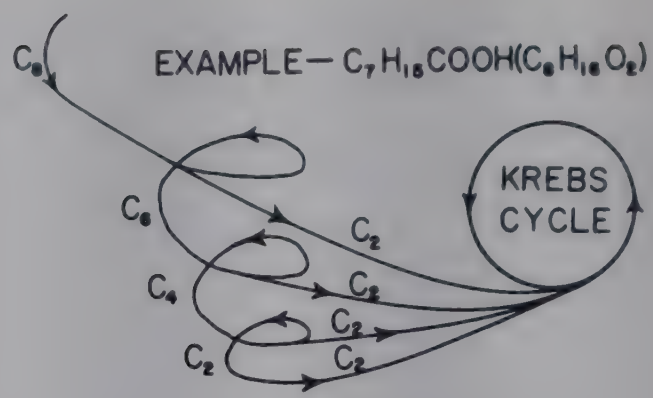


FIG. 7. THE FATTY ACID HELIX

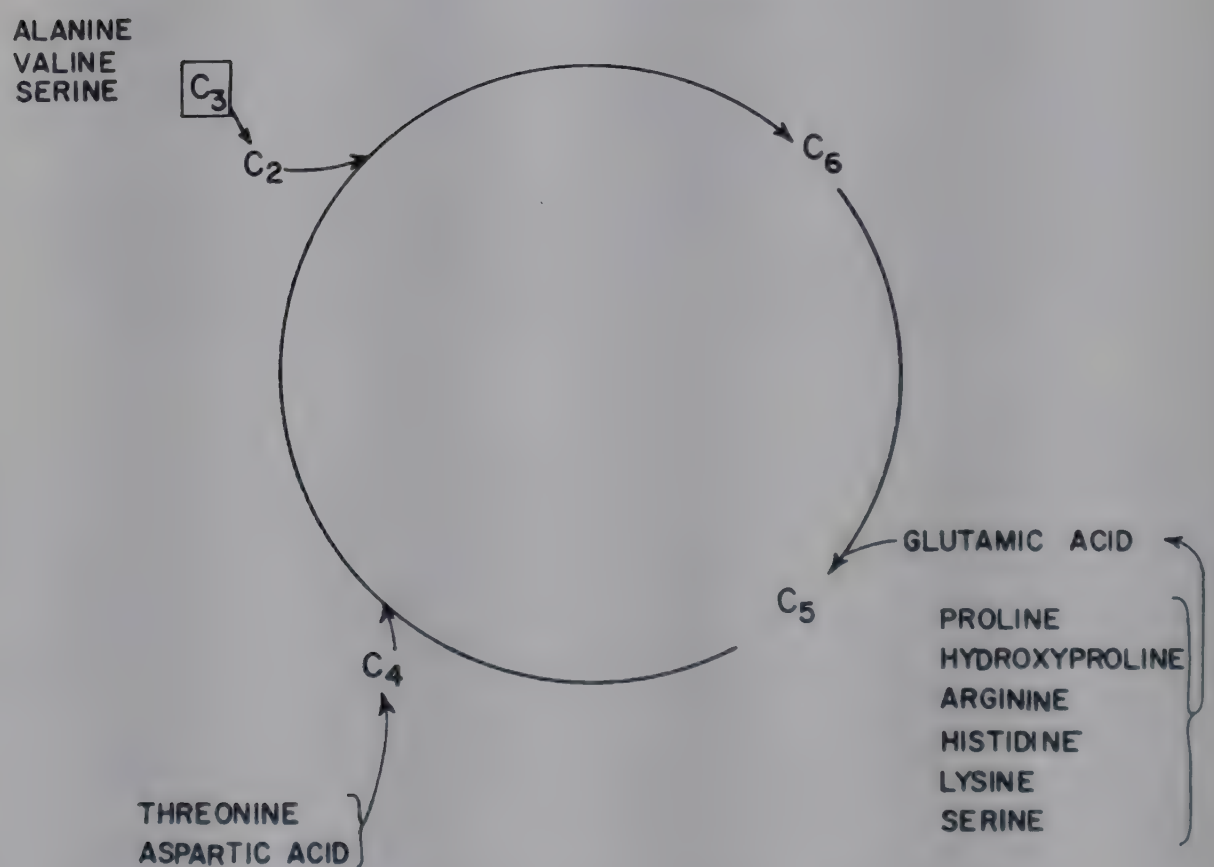


FIG. 8. PROTEIN BREAKDOWN AND THE KREBS CYCLE

through one of the "C_4" portals related to oxalacetic acid. The great bulk of the 20 or more building blocks of proteins can thus be utilized when eaten in excess (as in a steak, a quart of milk, etc.) for energy by the living organism.

MITOCHONDRIA AND ENERGY FORMATION

The foregoing paragraphs have emphasized the broad capacity of mitochondria to oxidize carbohydrates, fats and proteins, chiefly through the citric acid cycle as the terminal portion of each oxida-

tion pathway. Fig. 9 reviews these oxidations with the emphasis on energy production; this is the "grand act" of the Krebs cycle. As stated, close to 300,000 calories (300 kilocalories) of energy are obtained for each mole of pyruvic acid that enters the cycle. Since two moles of pyruvic acid arise from one mole of glucose, this means that upwards of 550,000 calories of energy are derived from each mole of glucose—about 80 per cent of the total energy available in the glucose molecule.

The mechanism by which the cell captures the energy from oxidation of carbohydrate or other foodstuffs is complex, and is not yet

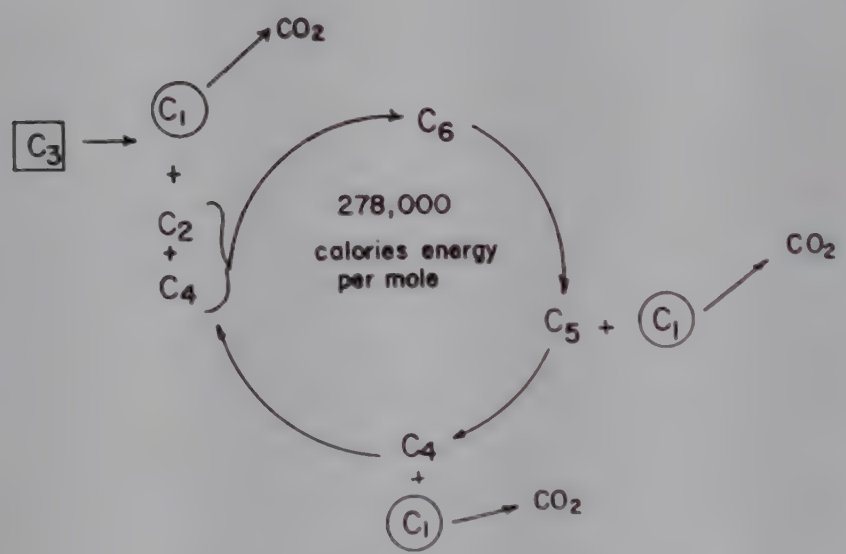


FIG. 9. KREBS CITRIC ACID CYCLE

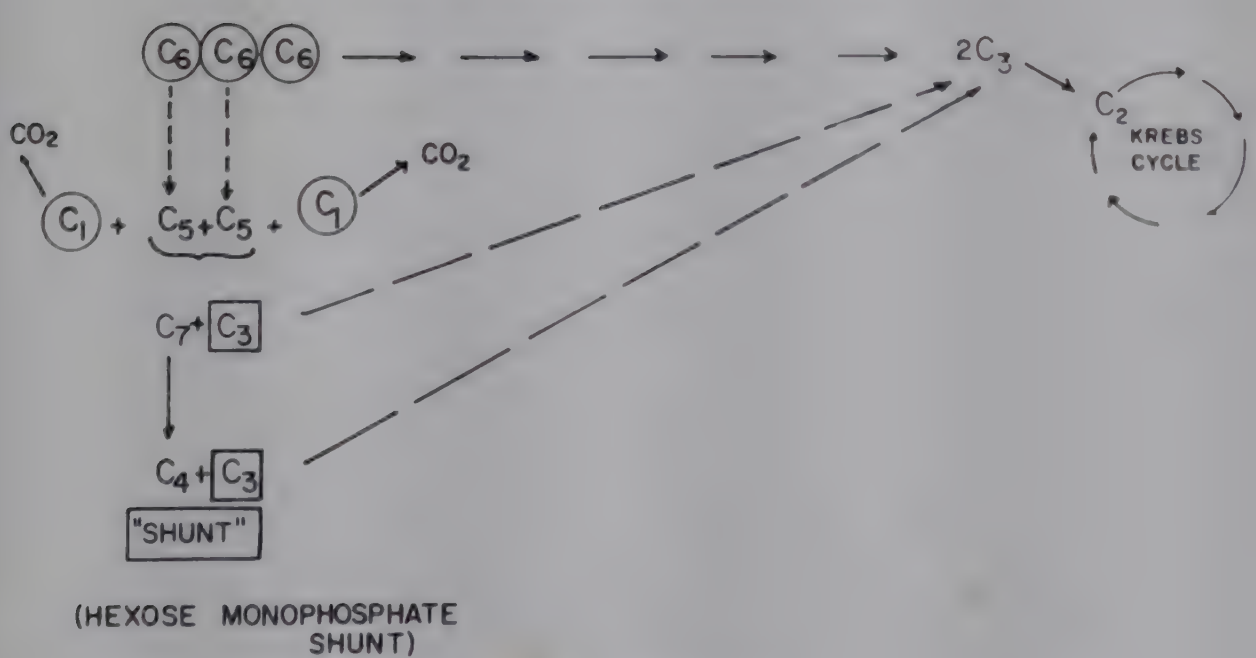
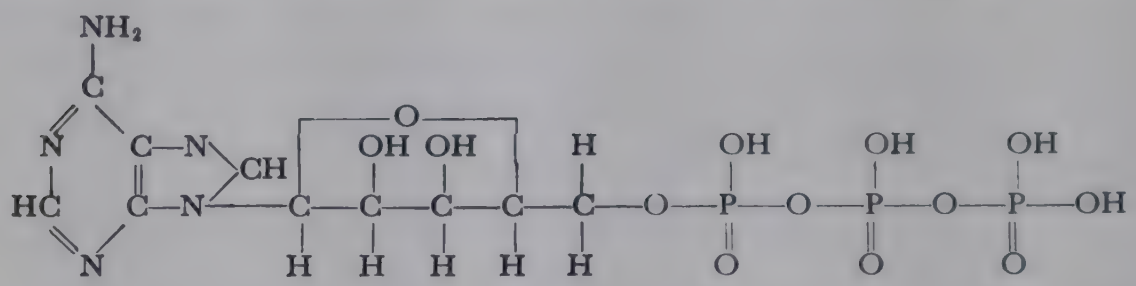


FIG. 10. GLYCOLYSIS

fully understood. Suffice it to say here that a specific compound is involved, adenosine triphosphate (ATP). Its structure is



This compound contains, in effect, extra energy in the two terminal phosphate groups. These are formed during the oxidation of food stuffs and constitute a reservoir of energy, which is easily available for other purposes, such as mechanical work, nerve transmission, or the many chemical reactions that are pertinent to the living cell and organism. When ATP is used as an energy source, it delivers this energy in individual "packets" at the rate of about 10,000 calories per mole, meanwhile losing its two terminal phosphate groups. The residual compound must then be "recharged" by oxidation of more foodstuffs, and so on. ATP is thus a currency of exchange so far as energy capture and transfer are concerned.

The enzymes that are responsible for this ingenious process of utilizing energy in living systems are, for the most part, localized in the mitochondria. It was this observation that led Claude to refer to the mitochondria as the "intracellular power plant" of the cell—where the fuel of the cell is burned and the energy made available for a host of normal cellular activities, many of which may transpire in the nucleus or in the soluble cytoplasm and thus are fed by the special energy packets provided in ATP formed during oxidation.

GLYCOLYSIS AND "DIRECT OXIDATION" OF SUGARS

Emphasis upon the Krebs cycle and the mitochondria has to this point been compelling, in demonstrating the close functional association between the visible morphological unit, the mitochondrion, on the one hand and the complex of enzymatic chemical activity on the other. This, together with continued discovery of the Krebs cycle in nearly all newly studied forms of life during the 1940's, led to the general view around 1950 that the Krebs cycle was *the* overriding oxidative pathway in the cells, especially for carbohydrates.

This point of view is summarized in Fig. 10, labeled "Glycolysis."

Glycolysis is the pathway by which sugars (mostly existing as glucose) are split to form a C_3 compound, pyruvic acid (compound *A* of Fig. 1). This breakdown comprises some twelve reactions, and enables glucose to decompose (via pyruvic acid) in the Krebs cycle. As expected, the enzymes of glycolysis occur in every organism where Krebs cycle prevails. The process, sometimes called the Embden-Meyerhof pathway in honor of two of the scientists who worked out its details, has been known in its entirety for about 24 years—approximately the same length of time as the citric acid cycle.

The horizontal series of reactions in Fig. 10 deals with glycolysis. (Glucose is labeled C_6 in this sequence.) The remainder of the diagram refers to another series of breakdown reactions of glucose, also known for some 24 years.

The vertical arrows in Fig. 10 represent the so-called "direct" oxidation of glucose. The first reaction steps were discovered by Warburg *et al* (1935), Lipman (1936), and Dickens (1936) working independently; through their efforts it was recognized that the six-carbon sugar, glucose, could be cleaved to a five-carbon (pentose) sugar in the organism, with one carbon atom disappearing as CO_2. This was an oxidation of a different sort from the Krebs cycle, and should in retrospect have triggered a more thorough search for the details of the process. That it did not was probably due to the nature of the main product, ribose, or more properly ribose phosphate. This sugar is found as a constituent part of ribonucleic acid, which is an essential component of the nucleus of every living cell in all kinds of organisms. Up to that time no satisfactory scheme had been worked out to explain the origin of ribose in the cell, and with the discovery that this obviously essential sugar could be formed directly from glucose, the needed explanation for the conversion seemed clear enough, and no further search for the details of the process was considered necessary.

Several years later it was observed in both plant and animal tissue extracts that two molecules of ribose phosphate could combine and rearrange according to the diagram in Fig. 10: $C_5 + C_5 \rightarrow C_7 + C_3$. In this abbreviated form of the reaction, the phosphate groups are neglected. However, the C_3 compound, glyceraldehyde phosphate, is a regular member of the glycolysis scheme, and it was assumed

by many that the C_3 unit was probably being formed as a means of "bleeding off" excess amounts of C_5 sugar that might be present, through oxidation of C_3 via the Krebs cycle. Soon thereafter it was found that the C_7 sugar phosphate (sedoheptulose phosphate) could also decompose, to a C_4 phosphate and another molecule of C_3 phosphate identical to the one formed in the previous reaction in Fig. 10. Again, the prevailing suggestion was that this was another step in the depletion of excess C_5—through C_7 breakdown, and the shunting into the Krebs cycle of another C_3 unit. The combined process was regarded as a shunt mechanism, and became known for a time as the *hexose monophosphate shunt.* The considered importance of the Krebs cycle as the final "prairie fire" of carbohydrate metabolism remained intact, even reinforced, by the "shunt" proposal.

THE PENTOSE CYCLE

The interesting observations concerning the interconversion of phosphates of C_7, C_6, C_5, and C_3 sugars, plus the appearance of a C_4 sugar, all seemingly representing breakdown products of glucose phosphate, prompted a number of workers to study the "direct" oxidative pathway more closely. It was not until 1953, however, that Horecker (1953) recognized the "shunt" in its true identity, not as a shunt pathway at all, but as a new cycle, an independent unit of metabolic machinery in which glucose could be oxidized completely to carbon dioxide and water without reference to either glycolysis or the Krebs cycle.

The major outline of the new cycle is given in Fig. 11. For convenience, six molecules of glucose-6-phosphate, labeled C_6P, are taken to represent the total pool of sugar used. We may evaluate the diagram more easily if we consider at first only three of these molecules, or the left half of the diagram. The initial product from each C_6P molecule is pentose phosphate (called C_5P), plus carbon dioxide. This simply restates the Warburg-Lipmann-Dickens discoveries referred to in the foregoing section. These are oxidative reactions, and can be regarded as the "direct" oxidative portion of the cycle.

Here a new set of reactions ensues, as shown in Fig. 11. Two molecules of C_5P that have arisen from two molecules of C_6P (in

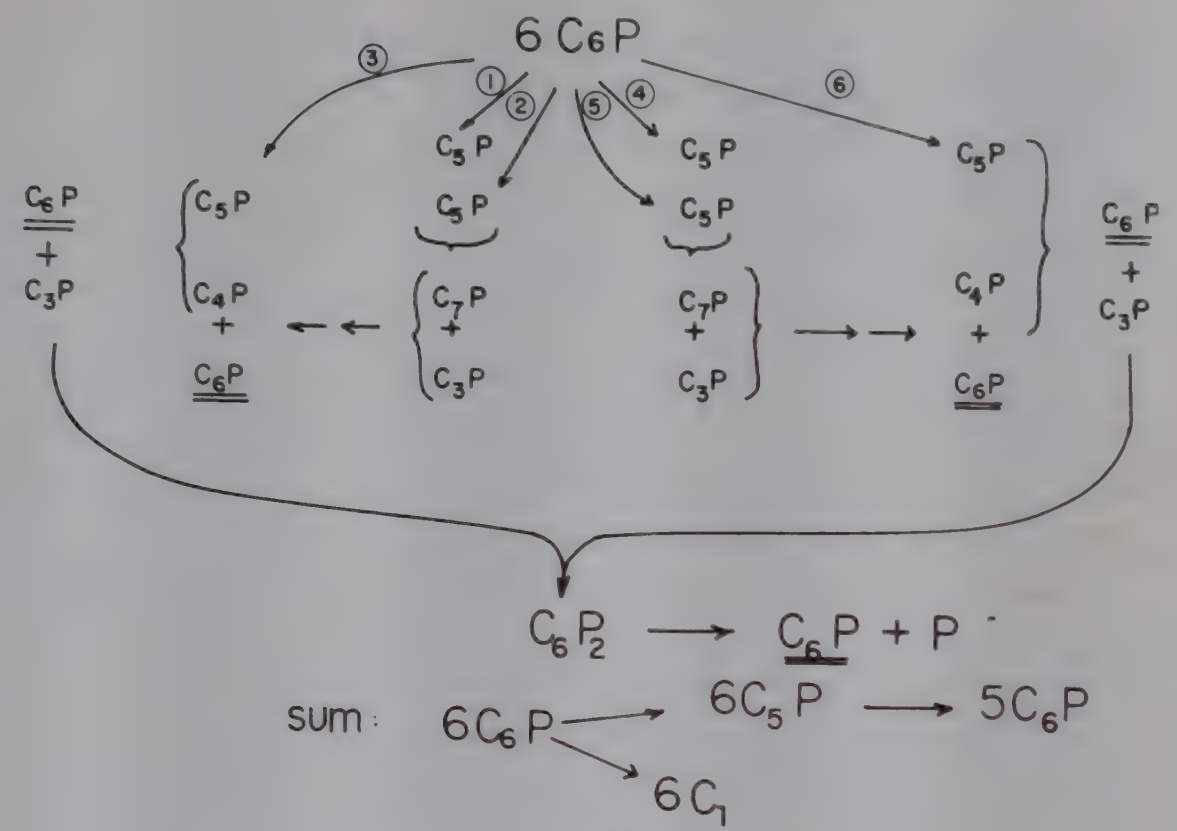


FIG. 11. THE PENTOSE CYCLE

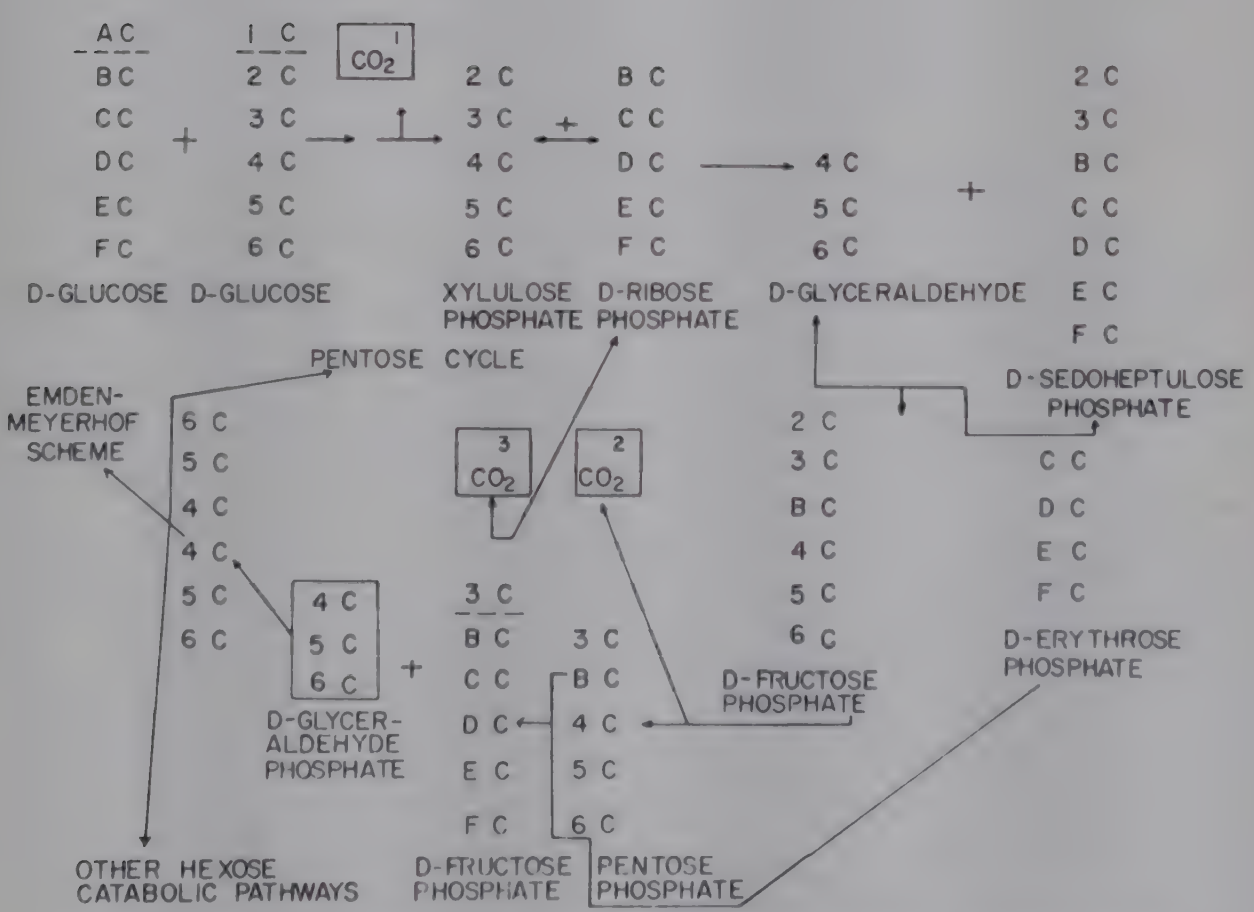


FIG. 12. FATE OF INDIVIDUAL CARBONS FROM GLUCOSE IN THE PENTOSE CYCLE

Reactions 1 and 2) undergo rearrangement, to produce C_7P and C_3P (sedoheptulose phosphate and glyceraldehyde phosphate). This reaction is promoted by the enzyme *transketolase*, which depends for its success upon adequate cellular concentrations of cocarboxylase, a derivative of vitamin B_1. The C_7 and C_3 units then rearrange further under the influence of a second enzyme called *transaldolase*, to form a tetrose phosphate, C_4P, *plus* a *molecule* of C_6P. The latter is in equilibrium with one of the C_6P's used at the start; so we see that a unit of starting material has been regenerated. Meanwhile, the C_4P that was also formed at this step is able to combine with a third molecule of C_5P (formed in Reaction 3 in Fig. 11); this $C_4P + C_5P$ union produced C_3P plus a *second* unit of the starting material, C_6P.

The net result of the activities on the left half of the diagram has thus been to engage three molecules of C_6P in a series of reactions that, in effect, regenerate two and one-half of them; a C_3P unit is left over, shown at the extreme left of the figure. By doubling the entire plan and inserting reactions 4, 5, and 6, the two C_3P units that would remain are joined as shown to form a hexose diphosphate, C_6P_2; the latter loses a phosphate group readily to form a *fifth* molecule of C_6P. Hence the sum:

$$6C_6P \longrightarrow 6C_5P \longrightarrow 5C_6P$$
$$\searrow\ 6C_1$$

This is a true cycle, since some of the starting material is regenerated at each turn. In our laboratory we have called it the *pentose cycle*, to emphasize the key position of pentoses in the scheme, and to deemphasize the earlier idea of a shunt, which this cyclic mechanism need not involve.

Fig. 12 indicates the fate of the individual carbon atoms in a hexose molecule as it traverses the respective steps in the pentose cycle. Two molecules of hexose are employed at the outset; one, in which respective atoms are labeled 123456, the other ABCDEF. The initial, oxidative steps result in cleavage of each hexose into a molecule of pentose and carbon dioxide; the latter represents the first carbon of hexose, and the pentoses thus correspond to the bottom five carbon atoms in hexose. The top two carbons of xylulose phosphate now become the top two carbon atoms of sedoheptulose

phosphate (transketolase reaction), and the triose phosphate formed corresponds to carbons 4, 5, and 6 of the starting glucose phosphate. The transaldolase reaction then returns three carbon atoms from sedoheptulose to triose phosphate, to produce a molecule of hexose phosphate with the sequence 23B456. The top carbon (carbon 2 of the original glucose) can be lost readily, since this hexose (fructose) phosphate can undergo oxidation after the manner of the glucose phosphate in the first reaction. The remaining pentose and tetrose phosphates rearrange, and another hexose (fructose) phosphate which loses its top carbon atom in the foregoing fashion; this time carbon 3 of the original glucose is lost, and a triose remains, with the sequence 456. Two units of this can combine, to give a hexose labeled 654456; and these atoms can appear as carbon dioxide after this variety of hexose undergoes recycling. Meanwhile, the *pentose* that remains at the end of the first cycle after the loss of carbon 3 from fructose phosphate, is labeled BCDEF—a sequence identical to that in one of the two original molecules of hexose added. In other words, according to this scheme one molecule of hexose ABCDEF has promoted the breakdown of 123456, and it would appear that one, or a few, molecules of ABCDEF could catalyze the decomposition of all the rest that might be present within a tissue. There are, of course, opportunities for dilution of the molecules listed, as for example on the right side of Fig. 12 where fructose phosphate is produced with the sequence of carbons 23B456. This species may be in equilibrium with the hexose originally added (sequence 123456) and there may therefore be considerable opportunity for mixing. The actual extent to which such mixing occurs has not been studied.

Occurrence of the Pentose Cycle

With the discovery of the pentose cycle and the recognition that it could operate quite apart from previously recognized carbohydrate degradation schemes, it became appropriate to seek the group of enzymes that catalyze these reactions, in a variety of living systems. This has been done in several laboratories, including our own, with the general result that these enzymes have been observed in every organism in which they have been sought. Thus, the existence of pentose cycle activity has been demonstrated in higher animals (Abraham *et al.* 1957; Black *et al.* 1957; Dickens and Williamson

1936; Dische 1938; Jolley *et al.* 1958 and 1959; Murphy and Muntz 1957; Newburgh and Cheldelin 1956; Racker 1954; Scott and Cohen 1951; Srere *et al.* 1955; Utter 1958; Villavicencio and Barron 1957), plants (Axelrod *et al.* 1953 ; Clayton 1959; Racker 1954; Silva *et al.* 1959; Srere *et al.* 1955; Utter 1958; Tabachnik et al. 1958), yeast (Horecker *et al.* 1956), other fungi (Newburgh and Cheldelin 1959), bacteria (Hauge *et al.* 1955; Kitos *et al.* 1958; Wang *et al.* 1958), and insects (Lehniger and Edsall 1951; Silva *et al.* 1959). In most of these investigations, activity of the enzymes in question appears abundant.

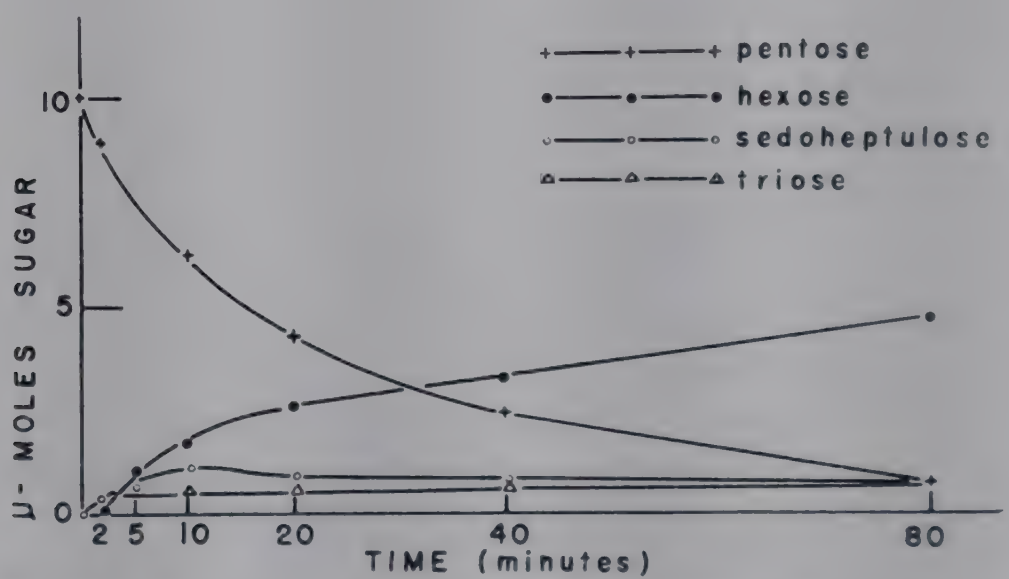


FIG. 13. DISAPPEARANCE OF ADDED PENTOSE (NON-OXIDATIVE) BY THE PENTOSE CYCLE

Cycle in soluble extracts of the pea aphid. Legend: +——+, ribose-5-phosphate; o——o, sedoheptulose-7-phosphate; Δ——Δ, triose phosphate; and •——•, hexose phosphate

The task of documenting an operating group of enzymes within an organism, such as those comprising glycolysis, the Krebs cycle, or the pentose cycle, is considerable. It generally involves (a) demonstration of extensive disappearance of any added intermediate in the pathway, with an accounting through analysis of the anticipated products, including carbon dioxide and water; (b) identification of each enzyme in the scheme, and demonstration of sufficient rates of reaction to coincide with the observed disappearance of added substrates in the intact organ or subcellular particles; (c) sufficient purification of the enzymes, and/or blocking of the pathway in several places to permit accumulation of expected products; and usually (d)

suitable experiments with isotopically labeled substrates to permit the investigator to determine the consonance between expected and observed labeling in intermediate products.

As might be expected, usually not all of these tests are applied to each system being examined. The effort is often considered too great to be warranted, and shortcuts are applied wherever they can be considered meaningful. Thus, for example, in Krebs cycle diagnosis a favorite test includes the reversible inhibition of succinate

TABLE 7

INTRACELLULAR LOCALIZATION OF PENTOSE CYCLE (NON-OXIDATIVE) IN RABBIT KIDNEY[1]

The reaction tubes contained 1 ml. of enzyme of the following fractions: S_{600}, 3.8 mg. of protein; $S_{25,000}$, 2.4 mg. of protein; $S_{105,000}$, 2.0 mg. of protein; the tubes also contained 200 μmoles of tris(hydroxymethyl)aminomethane buffer (pH 8.0), 100 γ of TPP, 20 μmoles of Mg, and 5 μmoles of R-5-P. Total volume 7.7 ml.; temperature 98.6 °F. 1 ml. aliquots were removed at various times and added to 1 ml. of 10 per cent trichloroacetic acid, centrifuged to remove protein, and assayed colorimetrically.

		R-5-P disappearing		Sedoheptulose formed		Hexose formed	
Fraction	Total Protein	μMoles Per Mg. Protein in Original Reaction Mixture	Total	μMoles Per Mg. Protein in Original Reaction Mixture	Total	μMoles Per Mg. Protein in Original Reaction Mixture	Total
	Mg.		μMoles		μMoles		μMoles
S_{600}	362	1.04	378	0.13	47	0.61	220
$S_{25,000}$	228	1.6	365	0.21	48	1.0	228
$S_{105,000}$	190	1.93	370	0.25	47	1.3	246

[1] After Newburgh and Cheldelin, 1956.

oxidation by malonate, in addition to demonstration of complete oxidation of all intermediates to carbon dioxide and water. A search for glycolysis in an organism often hinges on its conversion of glucose to lactic acid.

One of the most reliable shortcuts in pentose cycle detection involves, in addition to demonstration of glucose-6-phosphate and 6-phosphogluconate dehydrogenases, the non-oxidative disappearance of added pentose phosphate, accompanied by the transient formation of sedoheptulose phosphate and triose phosphate, and the slower accumulation of hexose phosphate. The curves in Fig. 13 illustrate this, where pentose is added at the start of an experiment to an extract of insect tissue (pea aphids) in the absence of air to prevent oxidation of the hexose formed. As expected, pentose disappears

while sedoheptulose phosphate and triose phosphate appear quickly, then level off at a lower concentration, whereas hexose phosphate accumulates steadily over the 80-minute span of the experiment. Over 80 per cent of the added pentose is accountable in the various conversion products. Additional experiments of the type outlined above would be required to establish rigorous proof of the operation of the pentose cycle in pea aphids, however this more "vigorous" method permits broader surveys to be carried out, with occasional, more meticulous checking for details of pentose cycle activity.

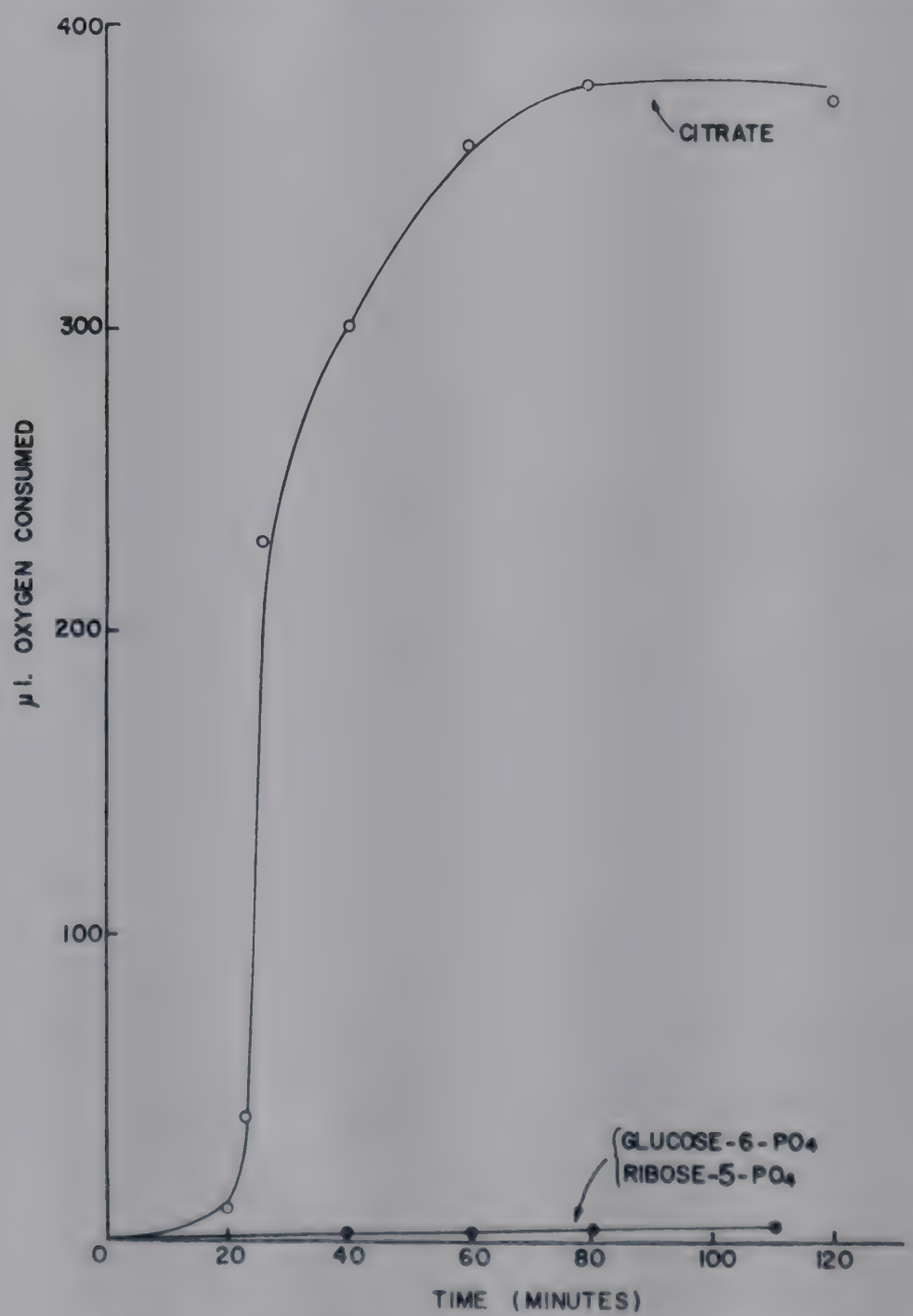


FIG. 14. OXIDATIONS IN RABBIT LIVER MITOCHONDRIA

Intracellular Distribution of the Pentose Cycle

Recognition of the widespread distribution of the pentose cycle enzymes in Nature, gave rise to the question as to the *intracellular* distribution of this complex: was it, like the Krebs cycle, located in the mitochondria? The answer to this question is provided by the data in Fig. 14 and Table 7 (see also Newburgh and Cheldelin 1956). In Fig. 14, a rabbit liver mitochondrial preparation is tested for its ability to oxidize glucose-6-phosphate and ribose-5-phosphate, both

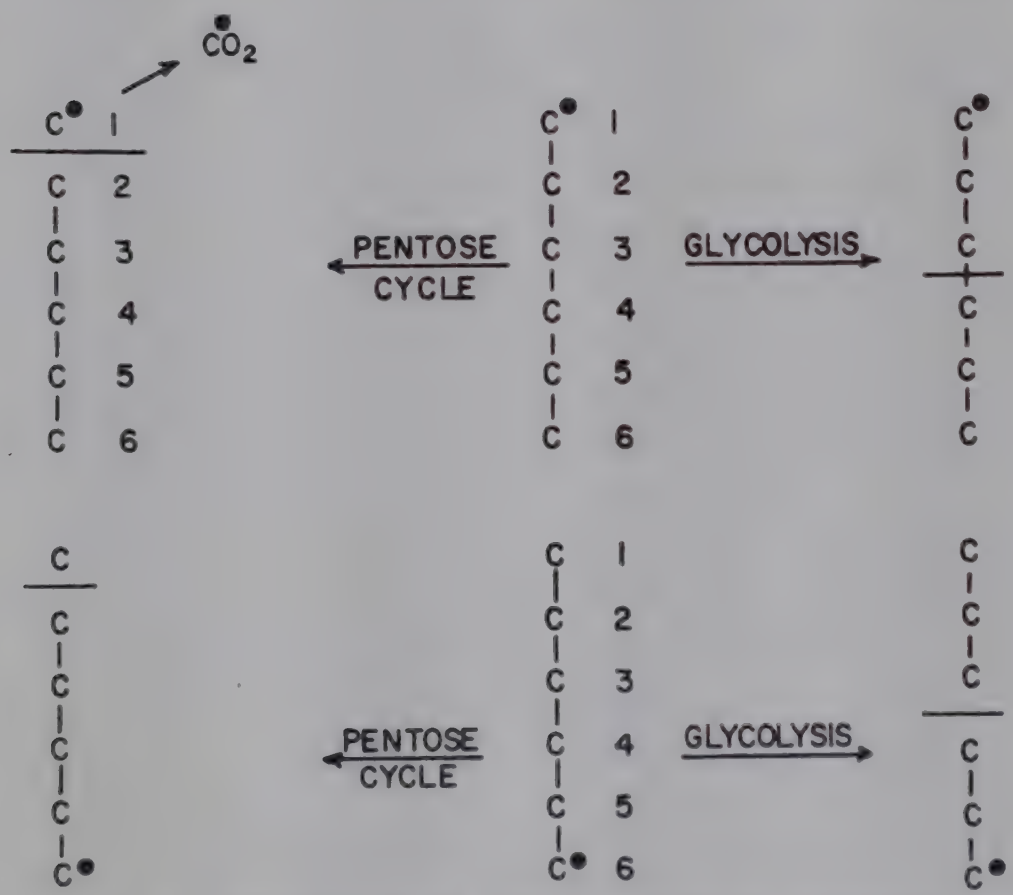


FIG. 15. DEGRADATION OF LABELED GLUCOSE BY DIFFERENT PATHWAYS

of which are components of the pentose cycle. Citrate oxidation is also included, as a positive control, since it is known to be fully oxidized by the mitochondria. Under the conditions of the experiment, about 390 μl. of oxygen were consumed in the oxidation of 5 μmoles of citrate (= approximately 70 per cent complete). Glucose and ribose phosphates, on the other hand, were not oxidized at all by the mitochondria, and it is clear that these oxidations must be sought from other fractions of the cell.

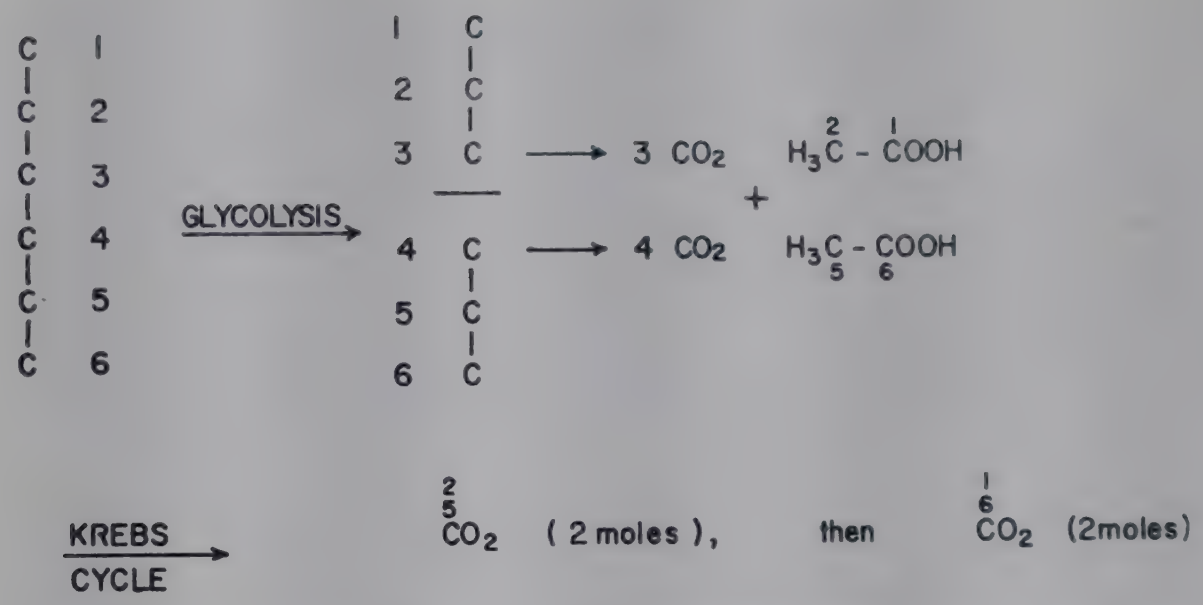


FIG. 16. DEGRADATION OF GLUCOSE BY GLYCOLYSIS AND THE CITRIC ACID CYCLE

Further partition of cell constituents was therefore made by centrifugation, into nuclei, mitochondria, microsomes, and the soluble cell "sap" that remained after removing all the particulate matter. The soluble fraction (after centrifuging for 2 hours at 105,000 × g) contained all of the pentose cycle activity (see Table 7), and none of these enzymes was found in the other portions of the cell. The contrast between these experiments and those relating to Krebs cycle activity is complete, and it is evident that these two major enzyme systems operate in quite different parts of the cell.

How Much of Each?

With the demonstration of near-ubiquity of both the glycolysis-Krebs cycle and the pentose pathways of oxidation in tissues, it was logical to ask "How much of each occurs in the normal, living cell?" This is a question that can be answered best through the use of glucose molecules as substrates, labeled in specific carbon atoms with C^{14}.

In principle the distinction appears simple, as outlined in Fig. 15. If glucose-1-C^{14} is fed to an animal and metabolized by glycolysis (and the Krebs cycle), the hexose chain is split in half. The yield of $C^{14}O_2$ will be the same as that from glucose-6-C^{14} because the two halves of the molecule are indistinguishable after cleavage and C-6 is indistinguishable from C-1. If, on the other hand, glucose-1-C^{14} and glucose-6-C^{14} are fed separately and metabolized by the pentose

pathway, carbon 1 is promptly split off from the remainder of the molecule. If this carbon is labeled, the respiratory CO_2 will be labeled; if not, it will not, at least at the outset of the experiment. Many organisms, when tested, promoted a faster conversion of carbon 1 to CO_2 than they did of carbon 6, and it was concluded by the earliest workers in this area that the new "pentose pathway" was quantitatively of great significance, sometimes more so than the glycolysis-Krebs cycle combination.

Actually, the picture is not quite so simple as this. If the pentose cycle is followed, the diagram in Fig. 12 indicates that carbon 1 will appear quickly in CO_2, followed in order by carbons 2 and 3; and carbons 6, 5, and 4 can appear in the respiratory CO_2 only after recycling. In glycolysis followed by the Krebs cycle, on the other hand (Fig. 16), carbons 3 and 4 emerge together, promptly, followed by carbons 2 and 5, then finally by 1 and 6 in equal proportions. The distinction was recognized a few years ago by the first workers in this field. However, the fact was at first overlooked that the rapid metabolism of carbons 3 and 4 might easily overshadow the conversion of carbon 1 to CO_2. Although the latter was richer

TABLE 8

RELATIVE CONTRIBUTION OF DIFFERENT PATHWAYS TOWARD TOTAL CARBOHYDRATE METABOLISM IN VARIOUS ORGANISMS[1]

Organism	Glycolysis + Krebs Cycle Per cent	Pathway, Entner-Doudoroff Per cent	Participation, Pentose Pathway Per cent	Reference
Capsicum frutescens longum (Pepper (fruit))	81	..	19	Doyle and Wang, (1958)
Tomatoes (fruit)	84	..	16	Barbour *et al.* (1958)
Periplanata americana (cockroach)	91	..	9	
Escherichia coli	72	..	28	
Saccharomyces cerevisiae	88	..	12	
Bacillus subtilis	65	..	35	
Aspergillus niger	77	..	23	
Penicillium digitatum	83	..	17	
Penicillium chrysogenum	77	..	23	
Streptomyces griseus	97	..	3	
Pseudomonas saccharophila	..	100	..	
Pseudomonas fluorescens	..	87	13	
Pseudomonas reptilivora	..	72	28	
Azotobacter vinelandii	..	..	100	
Acetobacter suboxydans	..	..	100	Kitos *et al.* (1958)

[1] After Wang *et al.*, 1958.

in C^{14} than the CO_2 from carbon 6 (thus measuring pentose cleavage activity), this was often found to be much less prominent in the total metabolism of an organism than was the $C_{3,4}$-metabolism which had traditionally been regarded as the "prairie fire" of terminal oxidation.

More recently, a kinetic approach has been employed, which compares the rates of conversion of glucose carbons 1,2,3,4, and 6 to CO_2. With this method, it has been possible to show that the pentose pathway (including the pentose cycle) seldom accounts for more than 25 per cent of the total carbohydrate metabolism in an organism, with the rest made up chiefly by glycolysis and the Krebs cycle. One major exception is the common vinegar producer, *Acetobacter suboxydans*, which appears to employ the pentose cycle for close to 100 per cent of its terminal oxidation of glucose to CO_2 (Kitos *et al.* 1958). The results of these investigations of different organisms are given in Table 8. In this table, the investigators (Wang *et al.* 1958) have also taken into account the possible participation of the Entner-Doudoroff (1952) pathway in total glucose metabolism by the various organisms. The latter pathway, outlined in Fig. 17, is like glycolysis in that when the six-carbon chain is split, two three-carbon moieties (pyruvic acid) are eventually produced, however the Entner-Doudoroff pathway is distinguished by virtue of the fact that the carboxyl group of pyruvic acid corresponds to carbons 1 and 4 of glucose instead of carbons 3 and 4 after glycolysis. This scheme has not appeared except in a few micro-organisms, however, and it appears likely that mammalian carbohydrate metabolism is generally well summarized by the two pathways described on the foregoing pages—the glycolysis-Krebs cycle pathway and the pentose route of metabolism.

The Function of the Pentose Cycle

Work in various laboratories has elaborated the identity and distribution of the pentose cycle, and has provided a measure of the share of the metabolic "traffic" that this pathway may carry. Remaining to be answered is the question of the major function(s) of the cycle, and it is here that considerable speculation may be offered.

There are several pieces of information in the literature which point to a *synthetic* role for the pentose cycle rather than one of

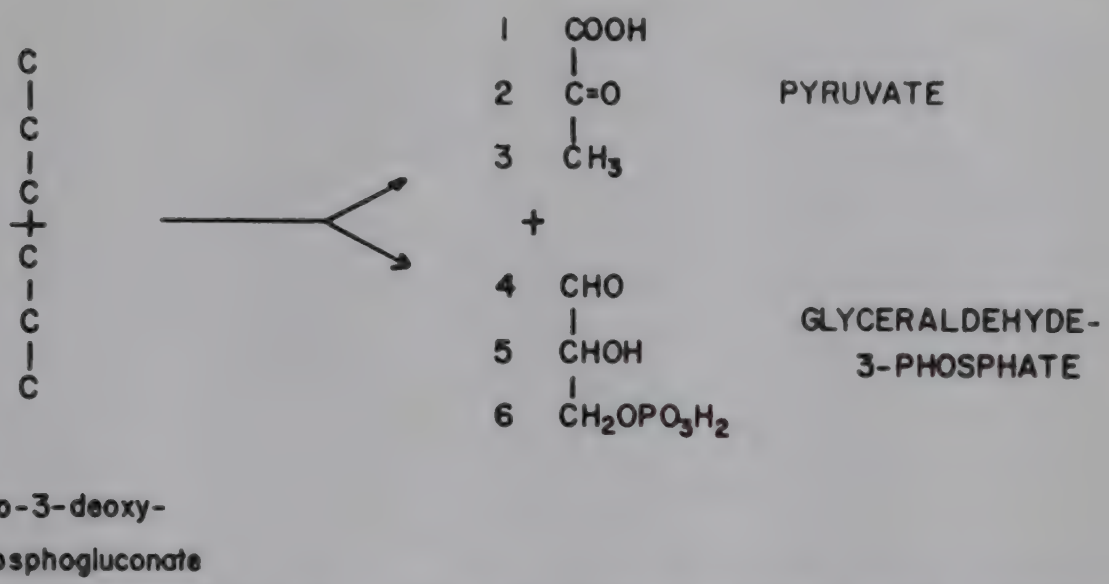


FIG. 17. THE FATE OF THE CARBON SKELETON OF GLUCOSE IN THE ENTNER-DOUDOROFF PATHWAY

carbohydrate oxidation, or breakdown, on a large scale. Some of these are: (a) the obvious need for ribose during growth, because of its occurrence in nucleic acids; (b) the finding (Jolley *et al.* 1958 and 1959) that the pentose cycle enzymes are more active and/or abundant during fetal development, and that in the final $^1/_4$ to $^1/_3$ of the gestation period, the fetus develops "adult" characteristics with respect to carbohydrate breakdown, i.e., the glycolysis-Krebs cycle route is greatly preferred in the latter stages as well as in post-partum life. (c) In higher plants, the key reaction of photosynthesis is one that involves a close relative of the pentose cycle, 1,5-ribulose diphosphate, as the primary aceptor of CO_2; the reactions that follow the incorporation of CO_2 involve both pentose cycle and glycolysis intermediates, *en route* to stored carbohydrate in the plant. All of these data point to the idea that the pentose cycle may function in many organisms primarily as a pathway for synthesis, even though *Acetobacter* and a few related micro-organisms may under appropriate conditions use this pathway extensively for carbohydrate breakdown.

SUMMARY

Our understanding of carbohydrate metabolism has undergone considerable change during the past quarter century, as the Embden-Meyerhof (glycolysis) pathway, the Krebs citric acid cycle, and the pentose cycle have successively emerged as major routes of

carbohydrate breakdown (or of synthesis, as in the green plant, from CO_2 and H_2O). Each of these schemes must be regarded as a major section of metabolic machinery; but the mere sampling of individual enzymes is not enough to establish their presence. Whereas the demonstration of an enzyme such as hexokinase was at one time considered *prima facie* evidence for the existence of glycolysis, we now recognize that with the exception of phosphofructokinase, all enzymes "belonging" to glycolysis are also characteristic of pentose cycle or Entner-Doudoroff operation. Only time and the industry of researchers in this area will determine whether overlapping may occur with another, as yet undiscovered, pathway of carbohydrate interchange. For the present, the three major routes mentioned above (glycolysis, Krebs cycle, and the pentose cycle) appear to suffice for carbohydrate metabolism in most organisms, and through the agency of labeled substrates we are able to approximate the importance of each in assessing their overall operation in carbohydrate metabolism.

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Z. I. Kertesz

Enzymes of Polysaccharide Synthesis and Degradation

This chapter deals with but a small corner of the enzymes involved in the formation and decomposition of polysaccharides of plant origin which are important in human food. No person attending this Symposium has to be reminded or convinced of the importance of polysaccharides in feeding this confused world's 2.8 billion people. Although cellulose is the most abundant polysaccharide, starch is the only polysaccharide of nutritional importance in human food. With the exception of a few minor groups like the Eskimos or the Masai in Africa, starch provides the major portion of food calories everywhere, whether it is consumed in bread, macaroni, taro root, rice, or potatoes. A third group of polysaccharides, the polyuronide pectins, do not provide calories for human beings but are important in providing our foods of plant origin with their typical and desirable structure. In addition, there is an ever-extending list of hemicelluloses, gums, and other polysaccharides isolated from plants but of lesser significance in the human dietary. In this review emphasis will be put on the enzymes involved in the synthesis and decomposition of cellulose, starch, and pectins.

The production of polysaccharides, as well as the rest of our food, depends on the enzymes present in biological systems. Enzymes or enzyme systems perform prodigious tasks of biological synthesis and catalyze the necessary reactions with high efficiency under remarkably mild conditions. These enzymes, or many of them, are present in our plants which serve as food directly and as raw materials in food processing, and are often active after harvest. They might catalyze useful or detrimental reactions, using as a criteria our selfish human desires. But often we can intentionally introduce more of the enzymes already present or enzymes entirely new to the system in order to cause desirable changes. As a consequence, there is now a multitude of enzymes produced commercially for use in food preparation and processing and for aiding people to utilize their food properly.

In dealing with polysaccharidases, even such a brief review must mention three separate factors which in recent years allowed tremendous increase in the rate of progress in our knowledge and understanding of both the polysaccharides and of the enzymes acting upon them. First, there was the development of macromolecular chemistry, the science dealing with giant molecules composed of repeating units, which is a comparative newcomer to our scientific specializations. Cellulose chemistry and the studies of artificial polymers used as plastics have contributed substantially to our understanding of the behavior of large molecules, although enzyme chemists seemed to have been slow in picking up this lead. In natural polymers such as the polysaccharides cellulose, pectin, and starch, we are dealing not only with large molecules but also with samples of variable heterogeneity. This realization provided a much firmer background for investigations of the enzymes acting upon these complex substances. Second, one must mention the now common use of radioactive isotopes. Third, there has been the development of the techniques of chromatography.

These, then, are the three major factors in the recent progress in the enzyme chemistry of polysaccharides. Thinking back to the days when these techniques were not available, one must indeed marvel at the accomplishments of those who were born too soon to apply them.

CARBOHYDRATE AND POLYSACCHARIDE FORMATION IN PLANTS

Although photosynthesis is the logical starting point of any discussion of polysaccharide formation in plants, it will be mentioned here only as an example of how repetition adds to the weight of an assumption. As those of us who went to school some years ago will remember, we were told (and the books said so) that formaldehyde is the primary product of photosynthesis in plants and that sugars are formed by the condensation of formaldehyde molecules. These assumptions were repeated so persistently that they have almost attained the dignity of facts. However, once the new techniques of using radioactive isotopes and chromatography became available, these hypotheses were soon discarded as erroneous. We know now that the nearest thing to a "first product" in photosynthesis is D-glyceric acid 3-phosphate, and that from this compound the plant carries out the synthesis of carbohydrates.

An additional fact which emerged from the labor of enzyme chemists during the past few decades should be emphasized. This is nature's ingenuity (or evasiveness) in being able to perform *through different pathways* syntheses leading to the same end products. Just half a century ago scientists were looking for "the" mechanisms of photosynthesis through which carbohydrate and polysaccharide syntheses or metabolism occurred. Today, it is clear that in most instrates the complicatedness and efficiency of natural processes which this certainly complicates the work of the researcher, it also demonstrates the complicatedness and efficiency of natural processes which allow living organisms to utilize various initial building materials for the same end.

The reversibility of enzyme action used to be a basic premise of enzyme chemistry. With reference to the biosynthesis of sucrose, the most important disaccharide, one might note that the long-assumed synthesis of sucrose in plants from glucose and fructose does not seem to be the pathway through which it is produced. To help explain this statement it should be noted that some bacteria contain a phosphorylase which, in the presence of phosphate, catalyzes the phosphorolytic decomposition of the disaccharide as follows:

$$\text{sucrose} \rightleftarrows \text{alpha-D-glucose 1-phosphate} + \text{D-fructose}$$

The reverse action produces sucrose through dephosphorolytic condensation of alpha-D-glucose and D-fructose with the production of inorganic phosphate. Evidence is now accumulating that sucrose synthesis occurs in plants by mechanisms involving a reversal of phosphorolysis rather than reversal of hydrolysis.

It also appears that the enzyme system responsible for sucrose synthesis in higher plants is not a simple sucrose phosphorylase of the type found in micro-organisms but rather that uridine diphosphate glucose (UDPG) is involved in the reaction as a D-glucose donor. Cardini, Leloir, and Chiriboga (1955) have shown that wheat germ, corn germ, and potato sprouts, among other tissues, contain an enzyme that catalyzes the reversible formation of sucrose from UDPG and D-fructose. Bean and Hassid (1955) confirmed this observation using the enzyme from pea meal and they have also shown that fructose may be replaced with xylulose (L-threopentulose), rhamnulose, or sorbose, to give the corresponding glucosyl disaccharides. But the fact that a synthesis *can* occur through a certain

reaction is no evidence that it indeed is *the* one or even *one* of the several reactions occurring in plants. Thus, even with a simple sugar-like polysaccharide like sucrose there is as yet some uncertainty about the mechanism of the reactions and about the enzymes involved in its biosynthesis in plants. This uncertainty is much more pronounced in the case of the non-sugar-like polysaccharides such as starch, pectins, and cellulose.

However, a master pattern for polysaccharide biosynthesis seems to be now emerging (Barker and Bourne 1953). The overall scheme, based on the work of many researchers, depicts polysaccharide synthesis as occurring in the following manner:

$$\text{Gt—O—X} + \text{H—O—Gr} \rightleftarrows \text{Gt—O—Gr} + \text{X—O—H}$$

where Gt indicates a sugar residue, X the aglycon portion, and Gr—O—H the receptor molecule. Each step in the synthesis of the polysaccharide involves the transfer of the group Gt— from —O—X to —O—Gr. A study of transfer reactions is also leading to a new concept of energy changes involved in the syntheses of polymer molecules as well as in the mechanism of the fissure of single glycosidic linkages. As Stacey (1954) states, this new concept underlines the significance of the term "transglycosylation" which has come into usage for expressing the type of change effected by the enzyme. Such reactions are in harmony with accepted views of enzyme mechanism, particularly if it is assumed that the polymer formation does not necessarily involve the direct step of exchanges of the glycosidic links between the substrate and product but rather is proceeding through the intermediate "glycoside-enzyme complex":

$$\text{Gt—X} + \text{enzyme} \rightleftarrows \text{Gt—enzyme} + \text{H—O—X}$$
$$\text{Gt—enzyme} + \text{H—O—Gr} \rightleftarrows \text{Gt—O—Gr} + \text{enzyme}$$

However, any general pattern of polysaccharide formation (if such exists) is still far from having been settled and there is no overall scheme which would give aid and comfort in such researches on the great variety of different polysaccharides encountered in nature. It is also of interest to note that "primers" initiating the polymerization reactions seem to be required for some syntheses while they are enhancing the progress of others. It is not even clear today if the requirement for a "primer" for one reaction but not for another actually only indicates the presence of primer-type impurities in some enzyme preparations. Certainly the role of "primers" in the

synthetic mechanism is a point about which we will hear more in the future.

Among polysaccharides, more research has been done on the enzymes involved in the synthesis and hydrolysis of starch than on almost any other group of enzymes. This seems appropriate considering the tremendous importance of starch in plant life and as human food. The realization that starch is composed of two glucans, namely, the linear amylose containing D-glucose units joined by alpha-1,4-linkages and the branched amylopectin which contains, in addition, 1,6 linkages at the branching points, helped much to clear the path for progress. A further factor was the already noted concept of macromolecular chemistry that all polymer samples represent heterogeneous mixtures made up of various molecular sizes, extents and kinds of branching, etc. Amylose and amylopectin, together with glycogen, were the first natural polymers for which a mode of biological synthesis became known. In harmony with what was said about different pathways, the synthesis of starch or starch-like polysaccharides is known to be possible through at least four mechanisms, entirely different from one another:

Starting Product	Enzyme System
Glucose 1-phosphate	Phosphorylase (P-enzyme) + isophosphorylase
Glucose 1-phosphate	Phosphorylase + Q-enzyme
Maltose	Amylomaltase
Sucrose	Amylosucrase

The phosphorylases act reversibly on either the glucose 1-phosphate or amylose, converting one to the other as equilibrium conditions dictate. Glucose 1-phosphate is converted to amylose with the release of inorganic phosphate while in the reverse reaction inorganic phosphate is combined with D-glucose during its liberation to give rise to alpha-D-glucose 1-phosphate ester. Such successive condensations can bring about the synthesis of linear amylose molecules of several hundred glucopyranose units. Amylose, the linear component of starch, can act as substrate for the branching enzyme or "Q-enzyme" to transform amylose into branched amylopectin.

While phosphorylase can completely degrade amylose, amylopectin can be only partially broken down by this enzyme. The resulting product is an enzyme-resistant "limit dextrin." In this process of

degradation the phosphorylase (like beta-amylase) evidently meets an obstruction at or near the 1,6-linkages in the polymer. To pass this hurdle, a special debranching factor (R-enzyme, isoamylase, or amylo-1,6-glucosidase) is required. This latter enzyme is found in beans, potatoes, yeast, and various other sources. The phosphorylases have been shown to occur in seeds, leaves, roots, the plastids and pollens of higher plants, and in many sources of animal and microbial nature. The potato is an especially good source of the phosphorylase.

But let us take a practical look at starch synthesis from the standpoint of food production and food processing. Do we know enough today to synthesize starch in practical quantities and with the required properties? The answer is "no." We have not even reached the point where we can satisfactorily explain the submicroscopic structure of the native starch granule. On the other hand, we have learned enough about agriculture so that we can enhance starch formation in various plants by providing the empirically established conditions which are most suitable for this process. But almost without exceptions, we do not know the details of the relationship between these physiological conditions and the increased efficiency of the enzymatic processes which partake in the synthesis of starch. With the abundance of starch in nature and with the comparative simplicity of producing crops containing high proportions of starch, there is little prospect that enzyme-catalyzed biosynthetic processes outside living organisms would ever be used for producing starch for food or feed, at least not for a long time to come.

In contrast with synthesis, the enzyme systems involved in the decomposition of starch are much better understood. The hydrolytic enzymes like the amylases and amyloglucosidases catalyze the splitting of glucosidic linkages. The equilibrium of these reactions is so far to the side of the split products that the reaction appears to be practically irreversible.

There are several different types of amylases and we will not enter into a discussion of the intricacies of this subject. It may be stated in an oversimplification that alpha-amylase breaks down the alpha-1,4-glucosidic linkages of amylose, but apparently is unable to split the 1,6-glucosidic linkages of branching amylopectin molecules. Beta-amylase splits only the second alpha-1,4-glucosidic linkage from the nonreducing chain ends. Simultaneously with the latter

action, the alpha-glucosidic configuration of the aldehyde carbon atom, which is involved in the hydrolysis, undergoes a conversion to the beta-glucosidic configuration. Thus, one molecule of beta-maltose is detached from the chain and then the enzyme acts again on the penultimate linkage in the same manner, removing another molecule of beta-maltose. Since beta-amylose is also unable to jump the hurdle of the 1,6-glucosidic linkages occurring at the branching points of amylopectin, the action will stop at these linkages giving the already noted "limit dextrins." The terminal 1,6-glucosidic linkages are split by the alpha-1,6-glucosidases (R-enzyme, isoamylase).

In contrast to the hydrolytic amylases, the already mentioned phosphorylases accomplish the degradation of starch by an entirely different mechanism. In this reaction there is a transfer of the glucosidic linkage from one radical to another. There is little or no change in the free energy with this reaction and, as a consequence, it is in most instances reversible. The name "transglucosidase" is now increasingly used for this type of enzyme. Phosphorylase can completely degrade amylose, but its action is stopped at the 1.6 linkages of amylopectin. So here again the debranching factor (alpha-1,6-glucosidase) is needed to allow complete hydrolysis. There are numerous other enzymes and enzyme systems involved in starch hydrolysis often pointing to still different mechanisms of decomposition.

Whatever is lacking on the theoretical side, our ability to induce and control enzymatic starch hydrolysis appears to be quite adequate. This is really not surprising when it is considered that starch-hydrolyzing enzymes have been used in practical production of food, like in bread baking and brewing, for at least 3,000 years. Starch-decomposing enzymes, the diastases, are used for a variety of purposes in the food industries and new and specifically more suitable enzyme preparations for given purposes continue to appear on the market. For instance, the fungal amylases aid in supplementing the low diastatic power of wheat flour and increase, in breadmaking, the availability of fermentable sugars. The bacterial amylases, which seem to be more temperature-resistant than fungal amylases, remain operative at much higher temperatures and thus provide special benefits in some instances. For the removal of starch from pectin extracts a diastase entirely free of pectin-decomposing en-

zymes must be used and such is provided by the enzyme manufacturers.

ENZYMIC SYNTHESIS OF DEXTRAN PRACTICAL

Perhaps one example where an enzyme is being used for the practical synthesis of a polysaccharide should be noted, even if the use of this product in foods is insignificant at the present time. Sucrose can be converted into dextrans by various species of *Leuconostoc* or by the enzymes isolated from these organisms. A 99 per cent conversion of the glucose present in sucrose may be obtained by the use of a pure enzyme (Hehre 1951). Phosphate plays no part in this synthesis, and the overall reaction does not seem to be reversible:

$$\text{sucrose} \rightarrow \text{dextran} + \text{D-fructose}$$

There are some indications from chromatography that the reaction might not be quite as simple as here illustrated. The structure of the dextrans can be controlled by the enzyme preparation used and by the type of initial receptor or "primer" added and some additional factors. A large inoculum enhances rapid production and determines the type of dextran produced. These synthetic polysaccharides are true dextrans from both the serological and chemical point of view. Whereas dextrans produced involuntarily have been bothersome for many years in the dairy industry, in sugar manufacture, and in many other operations, they now have been proposed for some uses in the food industries. One of these uses is for thickening syrups for chocolate candy centers (Wadsworth and Hughes 1946). Of course, the most prominent non-food use for partially hydrolyzed dextrans is as blood plasma substitutes in the treatment of shock.

ENZYMES OF PECTIC SUBSTANCES

Let us now turn to the little we know about pectin synthesis and to the enzymes acting upon pectic substances. The formation of the polygalacturonic acid skeleton of all pectic substances has been assumed to take place through the oxidation of the primary hydroxyl groups of galactans with a subsequent esterification of the carboxyl groups. It is now clear that this assumed genesis of pectin, so neat and simple, is most unlikely. There are some limited observations

by the incorporation of various isotope-tagged compounds and groupings into pectins, indicating possible pathways of synthesis. Radioactive carbon is incorporated into leaf and fruit pectin of the plum tree cultivated in an atmosphere containing $C^{14}O_2$ (Hough and Pridham 1956). Glucose-1-C^{14} also has been introduced into slightly immature boysenberries (Seegmiller, Axelrod, and McCready 1955). This compound and glucose tagged with C^{14} on carbon atoms 2 and 6, as well as galactose-1-C^{14}, were also introduced into strawberries (Seegmiller, Jang, and Mann 1956). Glucose and galactose seem to be equally suitable for pectin. In most instances some randomization of the C^{14} occurred but the intact hexose carbon skeleton was predominantly preserved.

When a tissue can utilize a given source of carbon or a sugar for polysaccharide synthesis, this is perhaps more a testimonial to the skill of nature and a demonstration of the ability of utilizing alternate pathways than it is evidence that the natural synthesis occurs in the plant through such steps. Whatever the case may be in this respect, we know practically nothing of the enzymes involved in the transformations which lead to the incorporation of the carbon supplied into the polygalacturonic acid (pectic acid) units which constitute the pectin skeleton. Leloir (Springer 1958) presented a hypothetical scheme of derivation of pectin through UDP-galacturonic acid. Smith, Mills, and Harper (1957) isolated UDP-galacturonic acid from Type I capsulated pneumococcus and Feingold and Hassid (1957) reported on the enzymatic formation of UDP-D-galacturonic acid in mung bean seedlings. While these observations are very significant, the question of polymer formation is still unsolved. Nor can one as yet disregard the possibility that pectins are produced under the control of a template.

Methyl groups of methionine tagged with C^{14} in the methyl groups are utilized in the formation of the methyl ester in pectins (Sato, Byerrum, and Ball 1957; Ordin, Cleland, and Bonner 1957; Nance 1958). Clearly, this is evidence of utilization or transfer but hardly proof that the naturally occurring methyl ester groups in pectins are normally derived from the methyl groups of methionine. The only conclusion which we can draw today is that we know little about the mechanism of pectin formation in plants and nothing about the enzymes which partake in these syntheses. Considering that pectins are present in all plant tissues, are quite clearly involved in the for-

mation of new tissue, and are well-recognized for their economic importance, this is indeed a major deficiency.

The situation is quite different from the standpoint of enzymes acting upon pectic substances. When we started our investigations of these enzymes in Geneva just 30 years ago, there were assumed to exist three enzymes acting upon pectic substances. These were an esterase, then called pectase, a hydrolase or polygalacturonase, then called pectinase, and an ambiguous enzyme or enzyme complex acting upon a mysterious compound called protopectin which thus far has resisted sharp definition and characterization. It was not long before this field of enzyme research, like so many others, underwent fragmentation and a series of discoveries indicating the existence of many distinct or at least significantly different enzymes.

With continued investigations the polygalacturonases have been separated into several subgroups. This brings to mind the scornful reception of our first report that more than one type of polygalacturonase might exist (McColloch and Kertesz 1948). In a classification recently presented by Deuel and Stutz (1958), polygalacturonases are classified into three major types although enzymes within any of these groups still may show variations in specificity and other properties. The three classes are:

1. Liquefying polygalacturonases which cause the fissure of the 1,4-glycosidic linkages characterizing pectic polyuronides in a more or less random manner.
2. Liquefying polymethylgalacturonidases which attack preferentially pectins of high degrees of esterification, and
3. Saccharifying polygalacturonidases which hydrolyze pectins only from one end of the chain molecule, probably from the reducing one.

Probably each of these enzymes requires a different structure around the hydrolyzable glycosidic bonds in order to form the active enzyme-substrate complex leading to fissure. Chromatography has been a most valuable technique in characterizing the mode of action of these various polygalacturonases and the sequence of events which take place as the large polymer molecule is eventually hydrolyzed into its basic monomer, D-galacturonic acid. The observations concerning the specifications of the three polygalacturonases show many unusual and puzzling facts and much more work is needed in order to understand completely their specificity and mode of action.

To turn for a moment to the pectinesterases (pectase, etc.) which

hydrolyze the methyl ester groups naturally occurring in pectin, the situation is somewhat more satisfactory. We know of their specificity with regard to their acting preferentially on both large molecules and methyl esters, and have considerable information on their behavior under a variety of experimental conditions. The differences found among pectinesterases of diverse origin are much less than is the case with polygalacturonases. Interestingly enough, although pectinesterase seems to be present in most higher plants, we have not the slightest evidence of a possible synthetic action of this enzyme. It is likely that a precursor or methyl group donor, as yet undiscovered, is involved in the synthesis.

Our knowledge of protopectinase and protopectin is most inadequate. Although we have several theories as to what protopectin might be, the only honest conclusion is that we do not know. So perhaps it is best if we say little about the enzymes acting on protopectin except that apparently some polygalacturonases and pectinesterases can attack protopectin. We know from hundreds of investigations that transformations in the pectic constituents (including protopectin) are involved in the quality and commercial value of many food crops, particularly fruits and vegetables. Yet, today we have not the slightest inkling of how any of these pectic changes occur in plant tissues, although we still assume that enzymes or enzyme systems are involved in some manner. This is one of the curious blind spots in our knowledge of plant life.

In contrast, commercial applications of pectin-attacking enzymes have now been well developed and such enzymes are being used on a large scale. We will mention only the extensive use of pectolytic enzymes in fruit juice and wine technology (Langlykke, Smythe, and Perlman 1952). Pectinesterases have been used for a variety of purposes of which we will note here only the production of low-ester pectins found so useful in the manufacture of low-sugar jellies, jams, and similar products.

ENZYME CHEMISTRY OF CELLULOSE

Cellulose is the most abundant of all naturally occurring organic compounds. It is present in practically all plant tissues and is also produced by micro-organisms. Cellulose has been utilized by people for a variety of purposes for thousands of years and is being commercially produced in vast quantities. It has been recognized as a

polysaccharide for over a hundred years and it is believed to possess the largest molecules among polysaccharides. Yet, we know practically nothing of the mechanism of cellulose synthesis in plants. Even our understanding of cellulose production by micro-organisms is inadequate and uncertain. The complexity of the problem is well indicated by the fact that cellulose produced by *Acetobacter xylinum* acting on D-glucose-1-C^{14} was found to have 82 per cent of the activity in C-1 of the D-glucose units and the remainder equally distributed between C-3 and C-4 (Minor, Greathouse, and Shirk 1955). This and additional evidence indicate that some of the glucose units must have been cleaved prior to cellulose formation.

The situation is little better as far as enzymes hydrolyzing cellulose is concerned. The classical source of cellulase is the digestive juice of the snail *Helix pomatia* but, of course, cellulose-digesting enzymes must be common in nature or we would be overwhelmed and covered by millions of tons of accumulated plant residues. Germinating seeds, fungal and bacterial extracts, and the digestive juices of crustacea and certain fish contain enzymes capable of decomposing cellulose. Enzymes for the utilization of cellulose by termites and ruminants are provided by the microflora in their digestive tract.

Celluloses derived from different sources show great variation in their susceptibility to enzyme digestion. This fact makes the use of carboxymethyl-cellulose (CMC) and other water-soluble cellulose derivatives of questionable value as substrates for the study of cellulases acting on native celluloses. Cellobiose and glucose are the end products of cellulose hydrolysis. Some authors regard the occurrence of glucose among the hydrolytic products as evidence that the disaccharidase cellobiase, distinct from cellulase, occurred in the enzyme preparation. Others suggest the existence of several different cellulose-hydrolyzing enzymes. A commercial preparation of a cellulase was available a few years ago but its activity was not great and apparently it found no extensive market. Yet, the potential for practical applications of different cellulases would seem promising. Much plant waste could be utilized in more economical and simpler ways if cellulose and hemicelluloses could be efficiently hydrolyzed into simple sugars by the use of enzymes. There is need for certain modifications of the properties of commercial cellulose

which perhaps could be attained by specific enzymes without materially reducing the strength of fibers. Pretreating certain foods or feeds with cellulases and hemicellulases would certainly provide some specific advantages. Enzyme treatments of various sorts have been extensively used for the modification of starches and pectins and it is disappointing that our knowledge of the enzymes acting upon cellulose is still so incomplete.

ENZYMOLOGY OF CELL WALLS AND PLANT TISSUES

Whatever we lack in our understanding of the action of enzymes forming and decomposing polysaccharides, it is eminently clear that these latter compounds, particularly cellulose, hemicellulose, pectins, and starch, are intimately involved in the structural architecture and functioning of plant tissues. We also suspect that some of these polysaccharides are converted from one type to another as a normal function of the living tissue. On the other hand, we know very little about the natural situation of the constituent polysaccharides in the plant cell structure and of the enzymes which synthesize, transform, and destroy them in the living tissue. One hoped that the appearance of electronmicroscopy would give an impetus to this field of histobiochemistry or cytoenzymology in which a fair start was made a few years ago. Unfortunately, there seems to be but an infinitesimal effort expanded in this direction (Holter 1952). One can have no illusion that this is an easy field of research. But progress in this field should parallel the advances in our knowledge of the enzyme action *in vitro,* whether dealing with pure enzymes or macerates. A better understanding of the structural location and functioning of polysaccharides and of the enzymes acting upon them *in situ* is imperative for the best utilization of our foods of plant origin. There is a great need for more systematic research in this difficult field.

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P. K. Stumpf

Lipids: Their Basic Metabolic Properties

In the past decade much information has been obtained by the biochemist concerning the metabolic interconversions of lipids. Today I would like to relate this knowledge to the general subject of this symposium.

As a food, lipids have quantitatively the best caloric value. Stored in large amounts as neutral, highly insoluble triglycerides, lipids can be rapidly mobilized and degraded for energy demands. Under oxidative conditions the cleavage of the highly reduced hydrocarbon chain releases four electrons for each C_2 unit formed. Presumably oxidative energy is trapped via the mechanisms of oxidative phosphorylation. We can then calculate, for example, that to produce one equivalent of ATP, two grams of palmitic acid must be combusted to carbon dioxide and water whereas, in contrast, nearly five grams of glucose are needed for an equivalent energy yield.

Lipids function also as important insulators of delicate organs. Nerve tissue, cell membranes, and the membranes of subcellular particles, and indeed the vital electron transport structures in mitochondria have as essential components lipids or their derivatives.

As we have already indicated, the chief storage form of available energy in the animal cell is the lipid molecule. When caloric intake exceeds utilization, excess food is invariably stored as fat. The body is frankly unable to store any other form of food in large amounts. For example, carbohydrates are converted to glycogen, but the capacity of the body to store this polysaccharide as a potential source of energy is strictly limited. In a normal liver the average amount of glycogen is about 5 to 6 per cent of the total weight while in skeletal muscle the glycogen content averages only about 0.4 to 0.6 per cent. Blood glucose, the glycogen precursor, is present at a level of about 60 to 100 mg. per 100 ml. of whole blood. Only under pathological conditions are these values drastically altered. The normal body very carefully regulates the carbohydrate concentrations in its various tissues by hormonal and metabolic controls. Thus carbohydrates serve only in a limited extent as storage forms of foodstuffs.

Proteins, the last major class of foodstuffs, differ considerably in their biological function from carbohydrates and fats since they serve as a source of the more than 20 amino acids required for *de novo* protein synthesis, and as sources of carbon skeletons essential for synthesis of purines, pyrimidines, and other nitrogenous compounds. Further, in an adult organism, where active growth has ceased, nitrogen intake is more or less geared to nitrogen output with the organism showing no tendency to store surplus proteins from the diet. These interrelationships are summarized in Fig. 18.

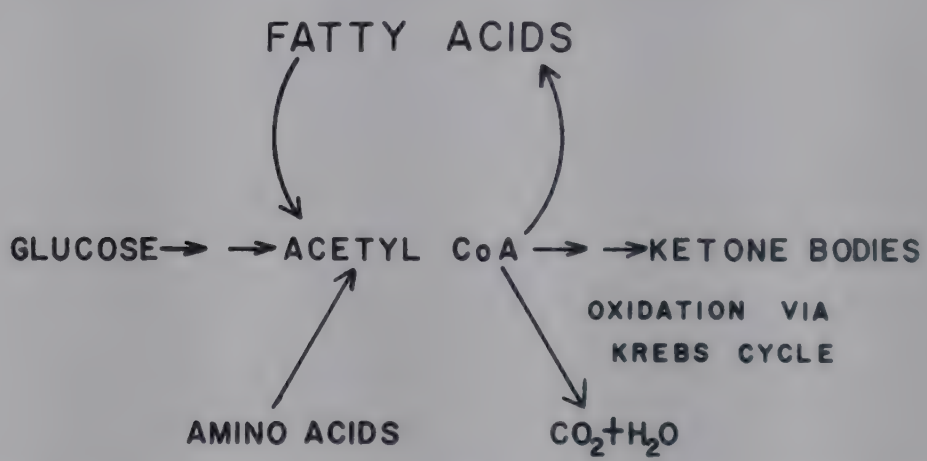
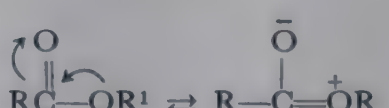
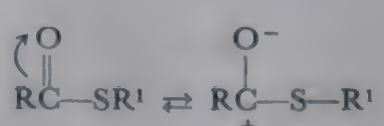


Fig. 18. Interrelationships of Foodstuffs and Acetyl CoA

The important integrating unit in lipid metabolism is of course the aforementioned C_2 unit, acetyl coenzyme A. A brief consideration of the chemistry of this intermediate is in order. This compound is a thioester and has its structural formula indicated in Fig. 19. Whereas oxygen esters have two resonating forms

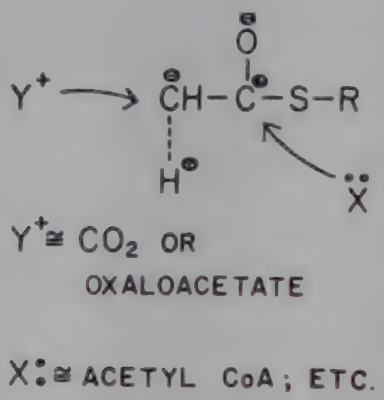


sulphur does not readily release its electrons for π bond formation and, therefore, thioesters do not exhibit the resonating forms of oxygen esters. Rather, thioesters exhibit considerable carbonyl character such as:



With a fractional positive charge on the carbonyl carbon, the hydrogen atom on the α carbon atom will tend to separate as a proton.

Acetyl CoA thus may participate in reactions in which the attack is either on the carbonyl carbon or on the carbon α to the carbonyl group:



With these basic facts in mind let us examine briefly the single most important biochemical system for the degradation of fatty acids, the classic β-oxidative sequence, first predicted by Knoop in 1904 and brilliantly elucidated by Green and his group at Wisconsin (Green 1954) and Lynen in Munich (Lynen 1954).

Fig. 20 depicts the series of reactions that describe the stepwise degradation of long chain fatty acids. Note the following features of the system:

(1) Only acyl CoA derivatives function as substrates.

(2) All enzymes associated with the system are localized in the mitochondrial particle in which is also localized the Krebs cycle and

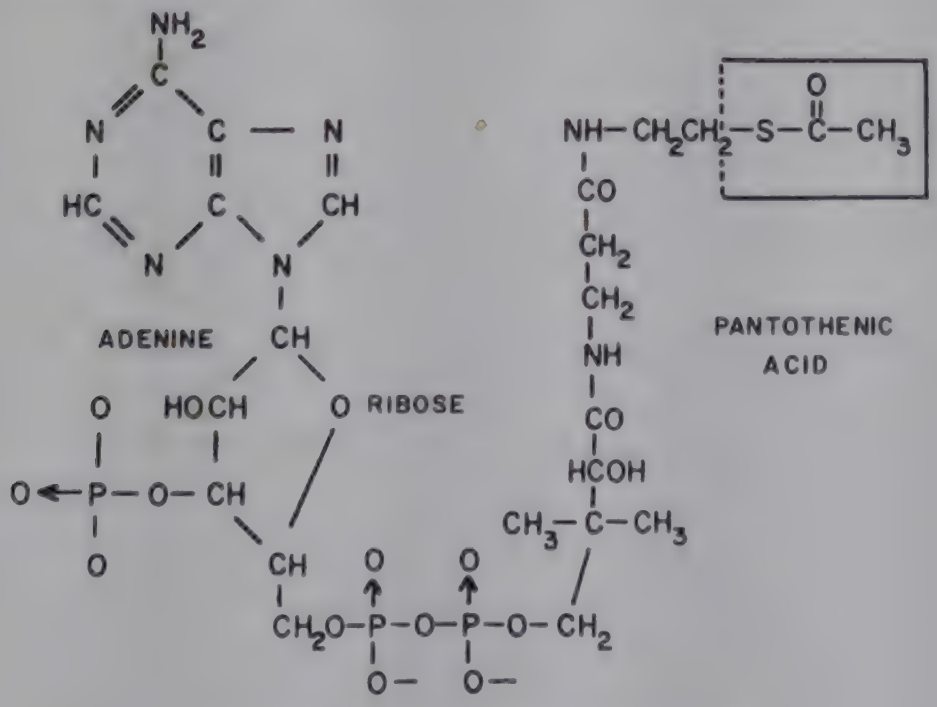


FIG. 19. STRUCTURE OF ACETYL CoA

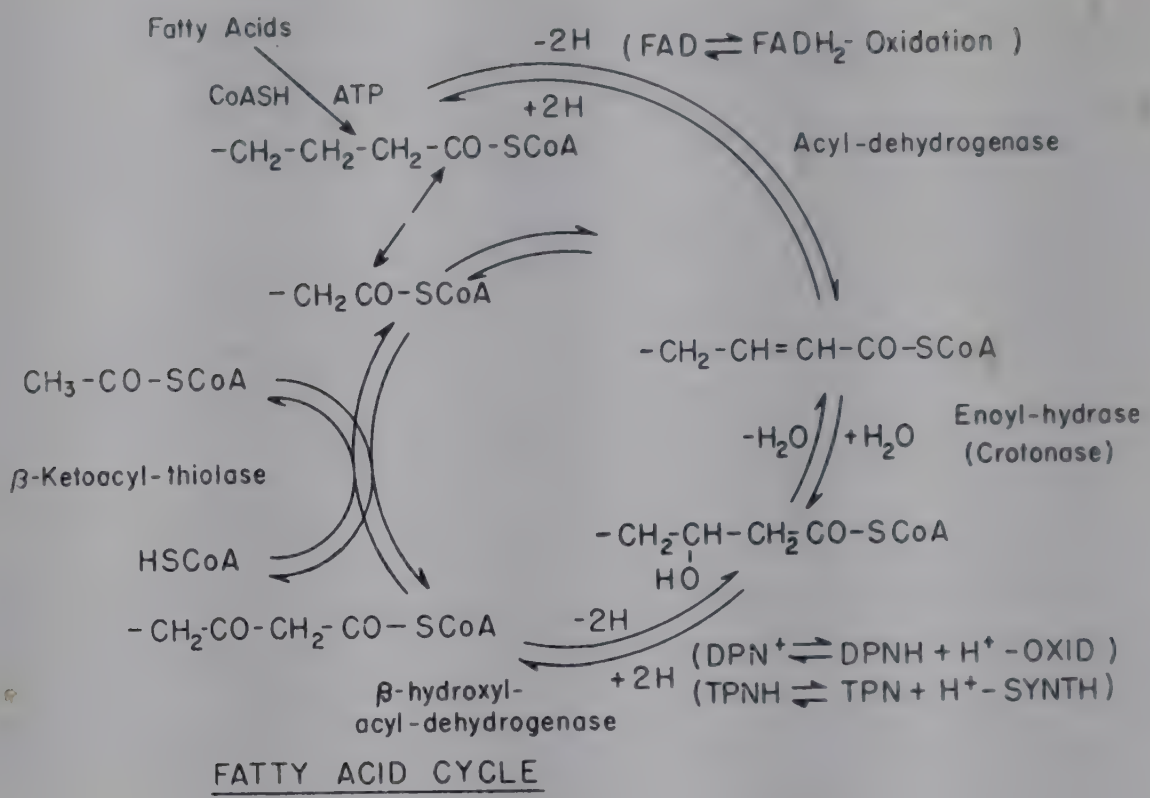


FIG. 20. β-OXIDATIVE SEQUENCE FOR FATTY ACIDS

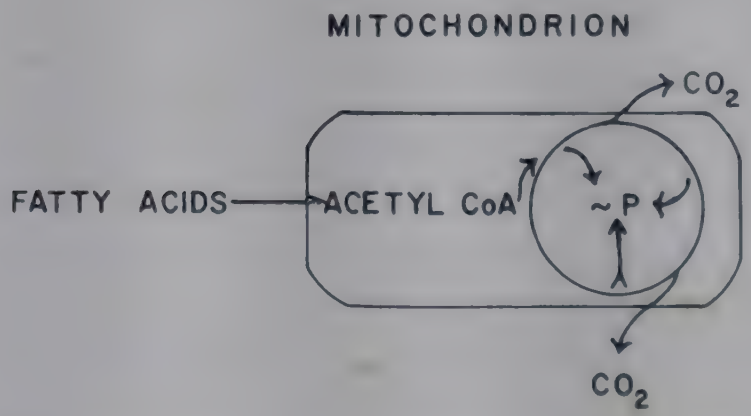


FIG. 21. RELATIONSHIPS OF OXIDATION OF FATTY ACIDS AND THE TRAPPING OF ENERGY IN THE MITOCHONDRION

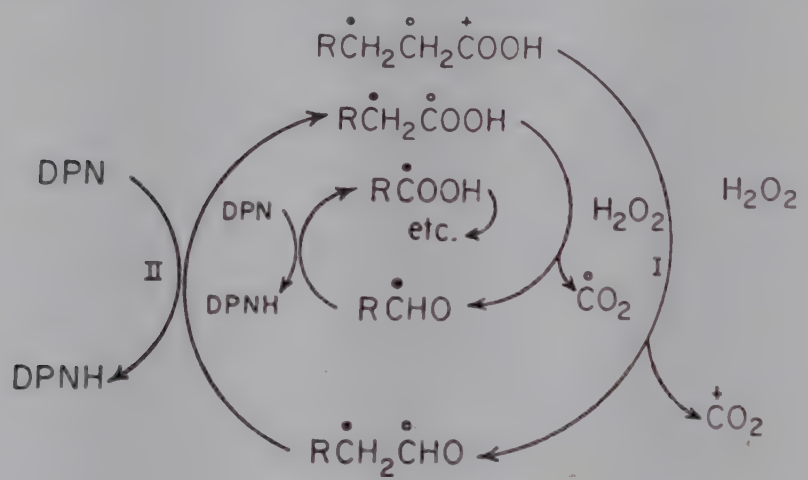
the oxidative phosphorylation sequence, both of great importance in the efficient transformation and trapping of the potential energy stored in the long chain fatty acid as in Fig. 21.

(3) The free energy of hydrolysis of a thioester is of the order of 8,000 calories, thereby placing this type of ester in the group called by Lipmann the "high energy or energy-rich type of bond." The driving potential built into coenzyme A esters is of great value in their many roles in biochemical reactions.

(4) Only one molecule of ATP is required to activate a fatty acid

for its complete oxidation, regardless of the number of carbon atoms in its hydrocarbon chain. In other words, whether we wish to oxidize a C_2 acid or a C_{18} acid, only one equivalent of ATP is needed. This makes for great economy and efficiency in the oxidation of fatty acids.

The β-oxidative sequence occurs in a large number of organisms, be they animal, plant, or bacteria. From the point of view of vitamin nutrition, we may note that riboflavin, pantothenic acid, nicotinamide, adenine nucleotide derivatives, and trace metals such as Mn^{++} and Mg^{++} play essential roles in the sequence. If there were



α-OXIDATION OF LONG CHAIN FATTY ACIDS

I Long chain fatty acid peroxidase

II Aldehyde dehydrogenase

FIG. 22. α-OXIDATION CYCLE IN HIGHER PLANTS

a deficiency in any one of these substances, serious blocks would result in the degradation of fatty acids.

Although the β-oxidative sequence plays the dominant role in fatty acid catabolism in animal cells, recent evidence suggests that in plants an α-oxidative sequence may participate in a limited oxidation of fatty acids (Stumpf and Bradbeer 1959). It can be shown that two enzymes participate in the stepwise oxidation:

(1) A fatty acid peroxidase which, in the presence of hydrogen peroxide, decarboxylates the carboxyl carbon of fatty acids to CO_2 while the residual hydrocarbon is converted to a long chain aldehyde.

(2) A DPN aldehyde dehydrogenase which oxidizes the long

chain aldehyde to the corresponding acid of the same chain length. This acid may then again enter the two step degradation sequence. This is summarized in Fig. 22. Note that here no requirement for coenzyme A, ATP, or metallic cations is demonstrated. The role of this new sequence in the total picture of fatty acid oxidation must await further investigation.

The function of lipoxidase, a highly reactive enzyme found in plants, which specifically oxidizes methylene interrupted polyunsaturated fatty acids such as linolenic and linoleic acids to hydroperoxides is as yet not clearly defined. Iron-containing proteins have also been shown to catalyze a similar oxidation in animal cells. Tappel (1959) has recently suggested that those cells with vitamin E deficiencies and hence a low antioxidant capacity, convert, by heme-catalyzed oxidations, polyunsaturated fatty acids to highly toxic hydroperoxides which may block important biochemical reactions. It may then be possible to implicate vitamin E in a more indirect but equally important activity in lipid metabolism.

As we have indicated earlier in Fig. 18, in a normal animal an excessive intake of carbohydrates leads to a rapid conversion to, and storage as, fat. When the β-oxidative spiral was finally elucidated and its enzymes separated into highly purified fractions, biochemists hastened to combine these isolated enzymes with acetyl coenzyme A and a reducing system. Although they searched for the appearance of long chain fatty acids, to their surprise, little if anything occurred. Since 1952 there have been reports (Brady and Gurin 1951) that a pigeon liver homogenate catalyzed the incorporation of acetate into long chain fatty acids in the presence of Mg, ATP, CoA, and DPN. Since then several investigators working with plant or animal homogenates observed similar results (Stumpf and Bradbeer 1959). In the meantime, intensive purification of a chicken liver preparation by Wakil and Ganguly (1959) of Wisconsin yielded a system which required as its essential cofactors, TPNH, ATP, acetyl CoA, Mn^{++}, and CO_2. The most surprising observation was that none of the β-oxidative enzyme activities could be detected in Wakil's preparations. Furthermore, one of the protein fractions of the chicken liver system required for synthesis showed a strikingly high concentration of protein bound biotin. Trace amounts of avidin completely inhibited fatty acid synthesis. It was then shown that for synthesis the

primary step is the formation of malonyl CoA from acetyl CoA and CO_2; this intermediate condenses with acetyl CoA to form a β keto dicarboxylic coenzyme A derivative. The several steps that have been proposed by Wakil are summarized in Fig. 23. Note that malonyl CoA is an ideal condensing unit since the presence of both the thioester group and the carboxyl group insures a highly reactive methylene group. Also the irreversible loss of the carboxyl group of the substituted dicarboxylic acid thioester favors the synthesis of fatty acids.

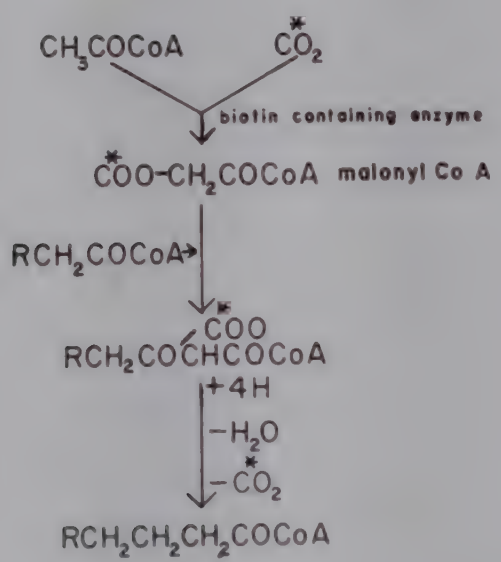


FIG. 23. ENZYMATIC SYNTHESIS OF FATTY ACIDS

The function of biotin is of unusual interest. It now appears that this vitamin is involved in carboxylation reactions. Lynen and coworkers (Lynen *et al.* 1959) have depicted the reaction sequence as follows:

(1) ATP + biotin-enzyme $\longleftrightarrow$ ADP-biotin-enzyme + P
(2) ADP-biotin-enzyme + CO_2 $\longleftrightarrow$ ADP + CO_2-biotin-enzyme
(3) Acetyl CoA + CO_2-biotin-enzyme $\rightarrow$ malonyl CoA + biotin-enzyme.

The ureido group in biotin is the functional site; biotin in turn is very firmly bound to the enzyme protein, presumably as a peptide bond with the terminal amino group of lysine in the protein chain.

One of the major problems in lipid biochemistry is the control or regulation of the anabolic and catabolic systems involving fatty acids. Biochemical solutions to these problems would throw much light on nutritional and pathological puzzles. It has been known for sometime that diabetic rats have lost the capacity to incorporate radioac-

tive glucose or acetate into fatty acids; the injection of insulin dramatically corrects the defect. In addition, the nutritional status of the animal greatly influences lipogenesis (Masoro *et al.* 1957). Thus a diet high in carbohydrates provides optimum conditions for fat synthesis. However, liver slices from fasted rats or those fed with a high protein or fat diet with an absence of carbohydrates have a very low capacity to convert radioactive glucose to fatty acids. Addition of substrate amounts of glucose corrects this deficiency. It has been assumed, therefore, that, in some manner, intermediates of glycolysis function in fat synthesis serving probably as a source of hydrogen to maintain TPN or DPN in the reduced state. A more intriguing solution to these results has been suggested by Catravas and Anker (1958) who have isolated a compound of unknown structure from

TABLE 9

VITAMINS AND FATTY ACID ENZYME SYSTEMS

Vitamin	Cofactor	Enzyme	Reaction
Riboflavin	FAD	Acyl dehydrogenase	Oxidation
Pantothenic acid	Co A	Acyl thiokinase	Activation
Nicotinic acid	DPN TPN	β Ketoacyl dehydrogenase enoyl reductase	Oxidation-reduction
Biotin	Biotin-protein	Carboxylases	Carboxylations
Thiamin	Cocarboxylase	Pyruvic oxidase	α Keto acid oxidation
Vitamin E	....	Stabilization of unsaturated acids	Antioxidant

yeast and livers of normal rats which has unusual physiological properties. Called lipogenin, it completely restores lipogenesis in intact fasted rats as well as in liver homogenates prepared from these rats. When injected into fed rats no stimulation occurs. Presumably in rats with a normal nutritional background lipogenin is present in optimum concentration but in the fasted rat the animal is either unable to synthesize the compound or destroys it, thereby interfering with the normal route of synthesis. With the basic knowledge now at hand concerning lipid synthesis, it will be of considerable interest to pinpoint the lipogenin effect with one of the enzymes involved in lipogenesis.

In conclusion, lipids have been briefly analyzed as a food, as a storage form of available energy; the known mechanisms of degradation and synthesis have been discussed and lipogenin has been implicated

as a new and important regulator in lipogenesis. In addition it has been pointed out that vitamin nutrition is of primary importance for the normal utilization of fats because riboflavin, nicotinamide, pantothenic acid, and biotin are key units of the cofactors responsible for electron transfer, carboxylation, and fatty acid activation (Table 9). If one of these vitamins is withheld from the diet the whole route of fat metabolism would be completely blocked.

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Ralph T. Holman

Enzymes and Polyunsaturated Fatty Acids

The polyunsaturated fatty acids (PUFA) which have non-conjugated double bonds occur widely in nature in both plants and animals. There are several distinct families of these acids, depending upon the position of the double bond nearest the terminal methyl group. Polyunsaturated fatty acids may have from 2 to 6 double bonds and the chain length may vary from 12 to 24 carbon atoms. The most abundant polyunsaturated fatty acids in plant matter are the C_{18} acids with 2 and 3 double bonds, and the most abundant polyunsaturated acids in animal tissues are the C_{20} and C_{22} acids with 4 to 6 double bonds.

These acids are subject to rapid oxidation by atmospheric oxygen, and are responsible for the rancidity which develops in fatty foods. They are also the active agents used in paint manufacture, because their autoxidation leads to the formation of tough water-resistant polymers. As a consequence of their easy oxidation and availability, much has been learned of their chemistry, but their biochemical role in nature is less thoroughly understood. Our present knowledge of the synthesis and metabolism of PUFA in living organisms is gained at the nutritional level, and very little is known about their function or metabolism at the cellular or enzymatic level of biochemistry. It is the purpose of this presentation to discuss the little that is known of the relationships of PUFA to enzymes, to point out consequences of enzyme action upon PUFA, and to speculate somewhat upon the function of these acids in metabolism.

LIPOXIDASE

The only enzyme system known to act specifically upon polyunsaturated acids occurs in vegetable matter. (For reviews of the subject, see Holman and Bergström, 1951 and Franke 1951). The enzyme was discovered via its action to oxidize a secondary substrate, carotene, and hence was erroneously called carotene oxidase before the primary action of the enzyme was known. Lipoxidase catalyzes the peroxidation of methylene-interrupted polyunsaturated

acids, and is not to be confused with other oxidases whose attack is at the carboxyl end of the molecule. The mechanism of action of lipoxidase is as shown in Fig. 24. The major products of the oxidation of linoleate by lipoxidase are similar to those obtained via autoxidation, except that the former are optically active (Privett *et al.* 1955), indicating an oriented rather than random attack by oxygen.

Although lipoxidase has been detected in many species and in several plant families, it is most abundant in legumes. Soybean

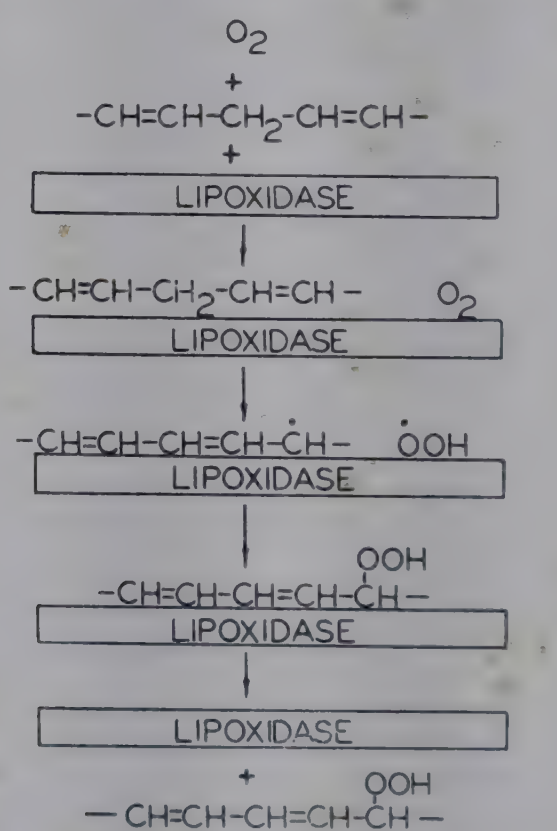
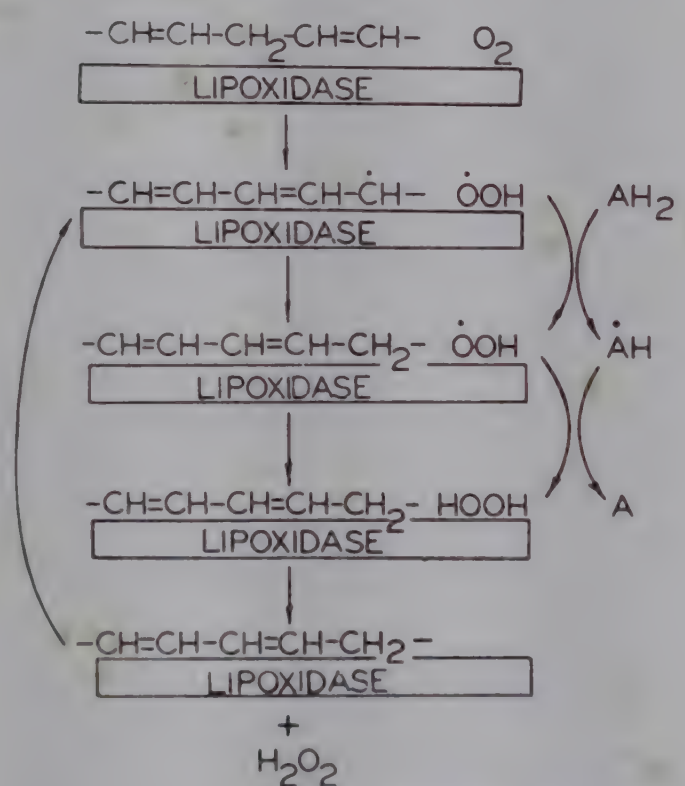


FIG. 24. MECHANISM OF LIPOXIDASE ACTION

lipoxidase is best known and characterized. Crystalline soybean lipoxidase (Theorell *et al.* 1947), judged homogeneous from electrophoretic, sedimentation, and diffusion patterns, has a molecular weight of about 102,000. It is a simple globulin whose amino acid analysis (Holman *et al.* 1950) accounts for 881 amino acid residues of the approximately 938 present. Leucine, isoleucine, and valine are relatively abundant, and provide areas of non-polar radicals capable of attracting the non-polar polyunsaturated acid substrates. The ultra-violet spectrum indicates the presence of some strongly absorbing component (at 280 mμ) not accounted for by the common aromatic amino acids present. The enzyme requires no cofactor, con-

tains no known prosthetic group, and no active metal. Its "active center" is not identified.

Lipoxidase is specific for substrates having two or more *cis* methylene-interrupted double bonds. It does not attack conjugated double bond systems, monoenoic acids, or polyunsaturated acids having *trans* double bonds. On the contrary, these are competitive inhibitors of lipoxidase. The enzyme is not inhibited by the usual enzyme poisons such as fluoride, cyanide, azide, or *p*-chloromercury benzoate (Holman 1947). The only inhibitors of its action are the



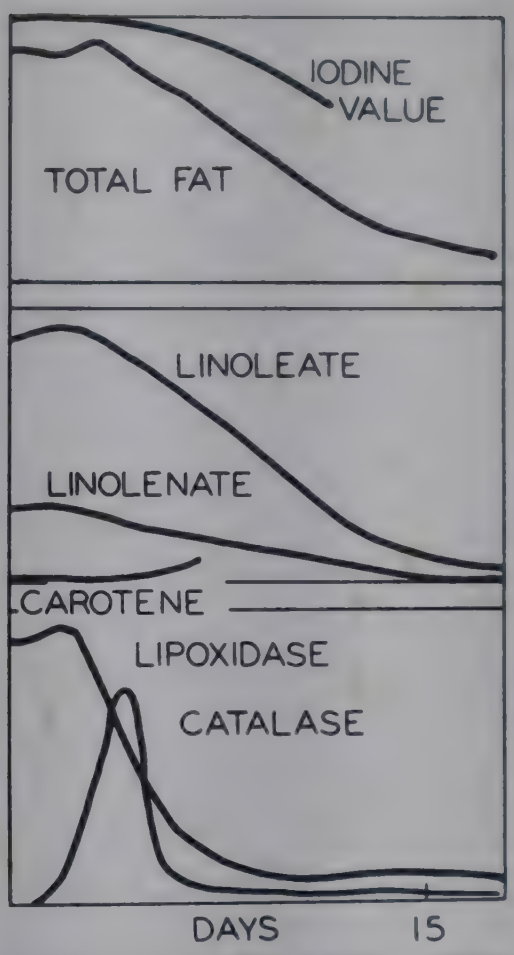
After Tappel et al. (1952)

FIG. 25. MECHANISM OF OXIDATION OF ANTIOXIDANTS AND OTHER SECONDARY SUBSTRATES BY LIPOXIDASE

antioxidants which also inhibit the autoxidation of its substrates. Under some circumstances lipoxidase can cause the oxidation of antioxidants and other secondary substrates without significant oxidation of the polyunsaturated acid substrates, probably according to the mechanism originally proposed by Tappel *et al.* (1952). (See Fig. 25.)

METABOLIC ROLE OF LIPOXIDASE

Extractable lipoxidase activity is high in the dormant soybean, but decreases sharply during germination, at that period when total fat



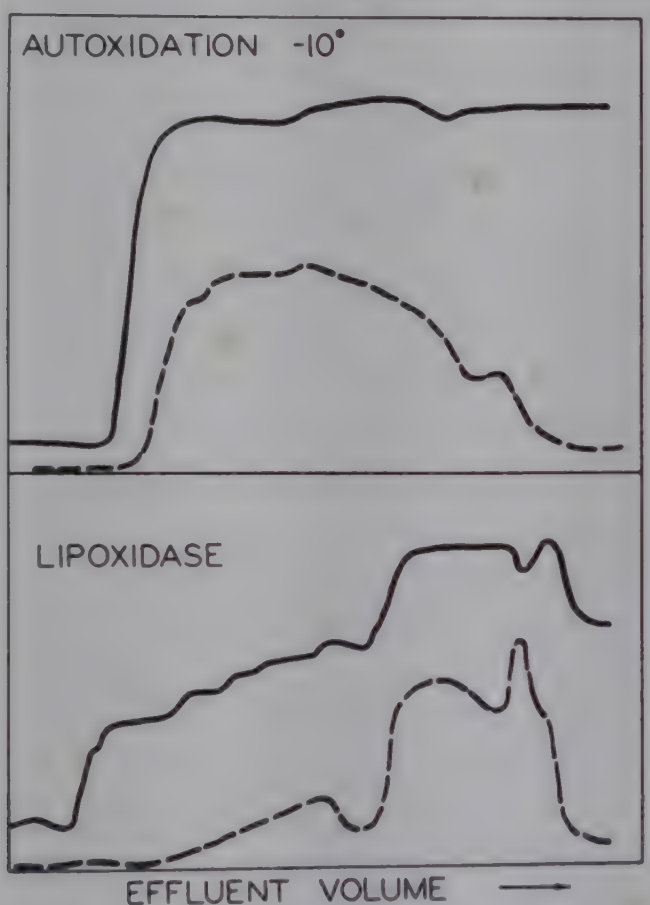
Redrawn from the data of Holman (1948)

FIG. 26. CHANGES IN COMPOSITION OF THE SOYBEAN SEED DURING GERMINATION

and linoleic and linolenic acids begin to decrease (Fig. 26). This coincides with a sharp and temporary appearance of catalase (Holman 1948). Catalase is known to utilize hydrogen peroxide for oxidation of such substrates as ethanol, and it is conceivable that it may function in a similar manner in the germinating soybean. If hydrogen peroxide is formed by the mechanism of Tappel *et al.* (1952), when a secondary substrate is oxidized by lipoxidase without concurrent oxidation of linoleate, it may be bound by catalase and used for the oxidation of another substrate. A shift of absorption spectrum of catalase accompanies its binding of hydrogen peroxide, and the spectrum reverts when the complex oxidizes a substrate. When lipoxidase and linoleate were allowed to react in the presence of catalase, the spectrum of the latter shifted characteristically indicating complex formation with linoleate peroxide, but the complex did not decompose.

During germination, the fat content of the seed decreases and the

carbohydrate content increases. It can be surmised that lipoxidase initiates the first step in the degradation of the polyunsaturated acids which comprise more than half of the fatty acid reserve of the dormant seed. What happens beyond the first step is not known. Perhaps catalase may play a role in the metabolism of the lipoperoxide. Crude soybean extracts cause the oxidation of linoleate yielding a



Redrawn from the data of Khan et al. (1954)

FIG. 27. DISPLACEMENT CHROMATOGRAMS OF PEROXIDIC PRODUCTS OF OXIDATION OF METHYL LINOLEATE BY AUTOXIDATION AT 14° F. AND BY LIPOXIDASE

Solid lines depict interferometric measurement of refractive index of effluent. Broken lines indicate light absorption at 2520 Å

larger variety of polar products than does autoxidation of linoleate (Khan *et al.* 1954, Fig. 27), suggesting that the soybean contains many other enzymes which have their action upon the hydroperoxidic product. Likewise, the carbonyl by-products of the oxidation, detectable by their absorption at 280 mμ are produced more abundantly the higher the temperature, and more abundantly by crude extracts than by crystalline lipoxidase. Koch *et al.* (1958) have found

strong evidence for two lipoxidase enzymes, one which acts upon free linoleic acid, the other upon triglycerides.

Although many investigators have attempted to detect lipoxidase in animal tissue, no convincing report of its presence has appeared. The catalytic activity in various tissues appears to be due to the presence of heme compounds (Tappel 1953) which cause reactions similar to those caused by lipoxidase and by autoxidation.

CONSEQUENCES OF OXIDATION OF POLYUNSATURATED FATTY ACIDS

Even at low levels of oxidation, the products of oxidation of PUFA and their subsequent degradation products impart objectionable flavors and odors even to the point of rendering the foods unacceptable. Any significant amount of oxidation in a foodstuff by lipoxidase, heme catalysis or autoxidation reduces the content of essential fatty acids (EFA), because the products of their oxidation have no biological activity as EFA. On the contrary, they have been found to have toxic properties when fed alone, and to be highly toxic when injected (Holman and Greenberg 1958).

Oxidation of polyunsaturated acids, from whatever cause, is accompanied by the destruction of fat-soluble and some water-soluble vitamins. The tocopherol content virtually disappears before organoleptically detectable rancidity appears, and vitamin A or carotene is destroyed in the early stages of the oxidation of a fat. Peifer (1954) has shown that the nutritive value of proteins is diminished when fed with oxidized fat, suggesting a possible oxidation of essential amino acids. The coupled oxidation of glutathione by pea lipoxidase has been demonstrated by Mapson and Moustafa (1955), and Dahle (1959) observed a cleavage of disulfide linkages by lipoperoxides. Thus both -SH and -S-S- groups are vulnerable to oxidative attack by lipid oxidation.

Lipoxidase has been observed by Muset *et al.* (1960) to be toxic when given in the drinking water, and when applied with linoleate topically. In both instances severe alopecia developed. They also found that lipoxidase plus linoleate injected intraperitoneally induced alopecia about the head and ulcerative lesions at the site of injection. The toxicity of lipoperoxides is shown more specifically by Ottolenghi *et al.* (1955) who observed that succinoxidase, amine oxidase and choline oxidase of rat liver mitochondria were inhibited

by lipid oxidation products whether these were induced by ultra-violet irradiation of the mitochondria or were added as irradiated linoleate or linolenate.

The involvement of lipoperoxides in pathologic processes is indicated by several observations. Lipoperoxides, which are not normally detectable in tissues, appear in adipose tissue when diets low in tocopherol and high in PUFA are fed. Lipoperoxides have been detected in atheroma and the substance called ceroid which is found in many pathologic tissues is probably a co-polymer of oxidized fat and protein. Maloney (1957) has demonstrated that the tumor-producing activity of Rous sarcoma virus was inhibited by the oxidation products of Rous sarcoma lecithin or by the oxidation products of linoleate or linolenate. Various inhibitory effects reported for unsaturated acids added to biological systems may well be due, not to the unsaturated fatty acid itself, but to its products of oxidation.

From nutritional studies it is known that linoleate is a precursor of arachidonate and a pentaenoic acid and that linolenate is a precursor of a pentaenoic acid and a hexaenoic acid. These conversions in the animal are potentiated by pyridoxine, but the enzymic mechanisms for the chain lengthening and the dehydrogenations have not yet been worked out (Witten and Holman 1952). The above observations made on a nutritional level have been verified for the conversion of linoleate to arachidonate by Steinberg *et al.* (1956).

POLYUNSATURATED ACIDS IN ENZYME SYSTEMS

Feeding a fat-free diet to animals induces a deficiency syndrome which can be prevented or cured by the essential fatty acid linoleic acid or acids related to it. EFA deficiency is manifested in anatomical changes, an altered pattern of tissue PUFA, an abnormally high basal metabolic rate, and respiratory quotient. Endogenous respiration of liver decreases, liver cytochrome oxidase and choline oxidase activities increase, and succinic dehydrogenase, glutamic dehydrogenase, and butyric dehydrogenase activities decrease. Factors which induce oxidation of PUFA, such as ultra-violet irradiation or the addition of oxidized linoleate or linolenate, also inhibit succinoxidase activity. These various observations suggest that polyunsaturated fatty acids are necessary components in some enzyme systems and that they are exposed and vulnerable to attack.

Recent data from our laboratory and the Enzyme Institute, Uni-

versity of Wisconsin (Holman and Widmer 1960) indicate that PUFA comprise a significant proportion of enzymatically-active mitochondria and subfractions thereof which retain specific enzyme activities. The PUFA content varied from about $^1/_3$ to $^2/_3$ of the total fatty acids present, and in one case amounted to 34.9 per cent of the total lipoprotein. Fractionation of the mitochondria into preparations possessing different enzyme activities did not segregate the several PUFA types. However, the observation is inescapable: polyunsaturated acids comprise a large proportion of the enzymes' lipids which thus far may not be removed from the protein without loss of activity.

Whether the polyunsaturated acids take part in the enzyme activity in the sense of a prosthetic group or active center is not known. Linoleate has been postulated as a prosthetic group for lipoxidase in the coupled oxidation of antioxidants and other secondary substrates. The polyunsaturated acids are sensitive to oxidation and could function in a reversible oxidation system, but this has not been observed experimentally. Polyunsaturated acids occur in phospholipids, cholesteryl esters, and triglycerides which are present in tissue lipoproteins as structural elements, and it may be that the vital role of PUFA is that they form a part of cytoplasmic structure providing cement or proper spacing between other components, and providing zones of non-polar matter capable of attracting non-polar substrates to an otherwise hydrophilic enzyme system.

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CHAPTER 7

A. K. Balls

Enzymes Affecting Proteins

As a general rule the amount of proteolytic enzymes that has been found in most kinds of food products is quite small. Proteinases are usually concentrated in glandular material. Fruits, vegetables and muscle are short on glandular tissue, except for the seeds. There are a few known exceptions to this statement, for example, the pineapple which is literally loaded—roots, fruit and foliage—with a variety of closely related proteolytic proteins. The latices of papaya and fig are also highly proteolytic, and some of the squash family including the humble pumpkin contain potent proteolytic enzymes. And yet it would be quite uncalled for to assume that in other products there is not enough proteinase present to have any effect, because these enzymes are very versatile catalysts. However, let us first review a few things about proteins.

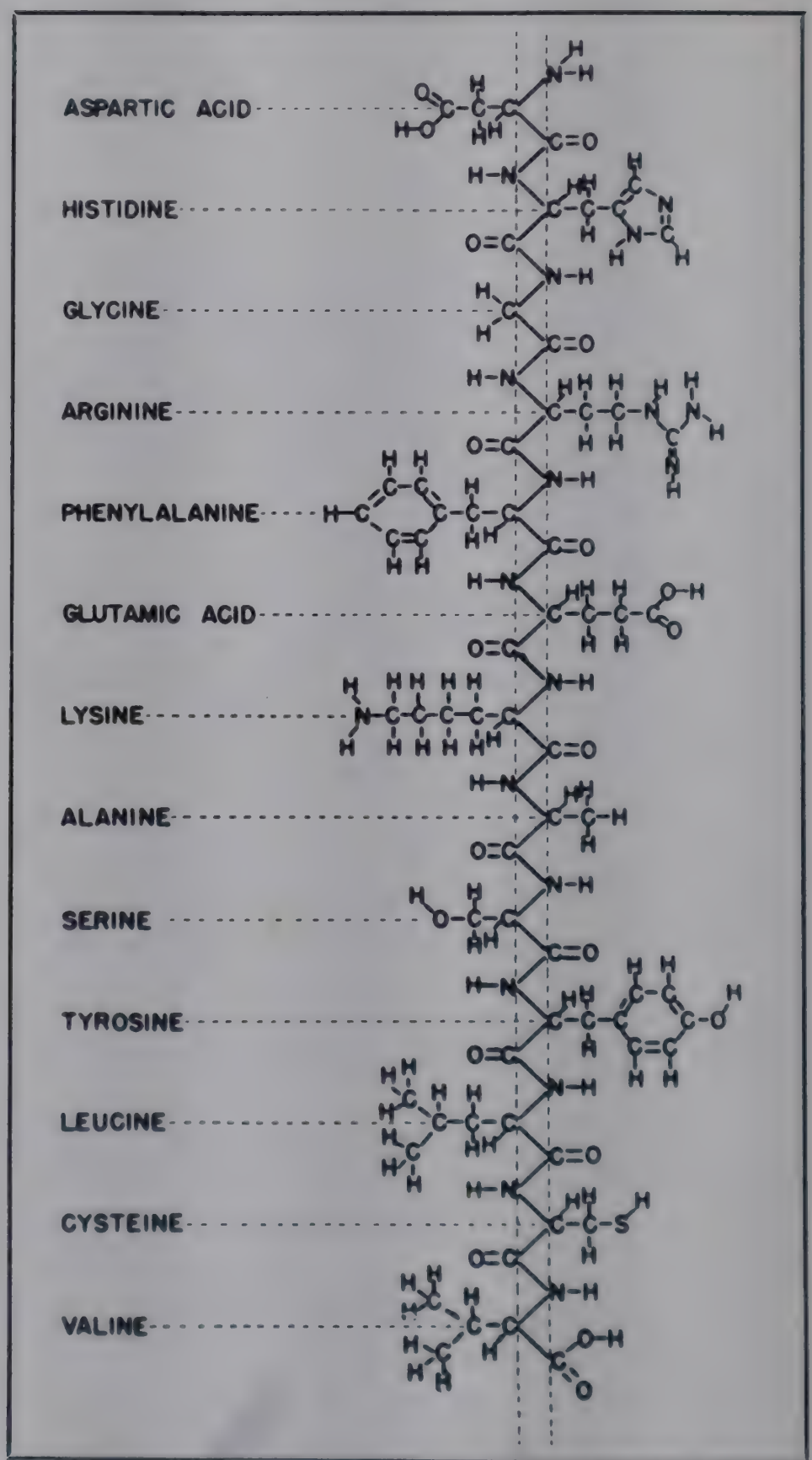
THE PROTEINS

To the best of our knowledge and belief, a protein is made up of amino acids joined in long chains by peptide linkages, that is CO-NH linkages.

Such a peptide chain has a sort of backbone as shown in Fig. 28 suggested by Lundgren (1945) of the Western Regional Research Laboratory.

The various side chains characterize the different amino acids. They stick out on the sides of the backbone and it is the special configuration of one or another group that attracts some particular proteolytic enzyme to that spot, where it speeds up the hydrolysis of the CO-NH bond in that vicinity, and thus breaks the chain in two (Fig. 29). This goes on repeatedly.

As these side-chains are different for different amino acids, it is easy to see why some proteolytic enzymes break the protein at one place and others at other places. Each proteinase has its own preference for the amino acids whose peptide bonds it helps to break quickly. Thus pepsin prefers to break a bond between two amino acids at least one of which is of highly acid character, while trypsin



After Lundgren (1945)

FIG. 28. DIAGRAM OF A PEPTIDE CHAIN

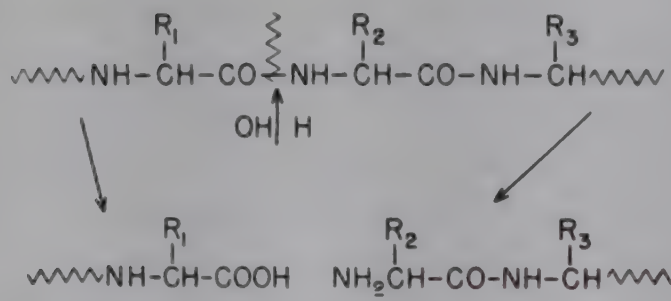


FIG. 29. DIAGRAM OF THE HYDROLYSIS OF A PEPTIDE BOND

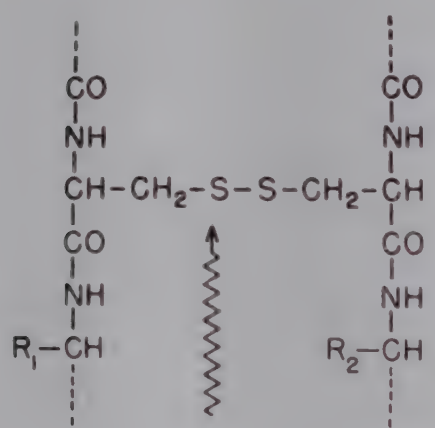


FIG. 30. DISULFIDE LINK BETWEEN TWO PEPTIDE CHAINS

prefers the bonds near an amino acid of basic character, and chymotrypsin prefers bonds that are in the vicinity of a benzene ring or some other bulky group.

Because of this it is possible to classify quite a number of protein-splitting enzymes into three distinct groups or types (Tallan *et al.* 1952) according to their resemblance to one of these three types (Table 10), but the enzymes in each group are not the same, nor necessarily all alike.

Another way to divide the proteinases is based on whether or not the protein must contain an —SH group in order to be active. There are thus sulfhydryl proteinases and the other kind. The two schemes of classification appear to overlap. It might be remarked that in the animal organism the only sulfhydryl proteinases so far recognized (two of the cathepsins) have been found within the tissues, as contrasted to the digestive enzymes, which are in fact excretory products. Because they are intracellular enzymes, the cathepsins may well merit the greater interest on the part of food technologists.

In native proteins, that is in proteins as they form in nature, these long peptide chains come packed together parallel to each other, and probably in bundles of parallel pairs. The chains are loosely

bound together by hydrogen bonding and also by an occasional sulfur linkage; half of a cystine molecule being in one chain, the other half in the chain parallel to it. So the two chains are held together here and there by the two sulfurs of cystine (Fig. 30); half of this amino acid then belongs to one chain, the other half to its parallel mate.

In addition, parallel pairs of amino acid chains are thought to be coiled together in a spiral, like railroad tracks winding around and around a hill, or else they are packed together in layers (See Neurath *et al.* 1956). There are, in fact, two kinds of proteins: fibrous and globular.

It is evident that some of the side-chains of the several amino acids are going to be on the inside of such a structure. Being inside, they will not be exposed where a protein-splitting enzyme can attach to them. If something happened to separate these parallel strings or

Table 10

BONDS PREFERRED BY THREE CLASSES OF PROTEINASES

Pepsin Type	Trypsin Type	Chymotrypsin Type
Between acidic amino acids, e.g. tyrosine, phenylalanine, glutamic, aspartic, cystine	Between basic amino acids: arginine, lysine, histidine, and neutral amino acids	Between amino acids with bulky side-chains (e.g. tyrosine, phenylalanine) and (probably) any of the others.

layers, there would be many more sites for the enzyme to attack. The protein would then be much more quickly and also more completely split apart by enzymatic action. When such a separation is made the protein is said to have been denatured. This picture, oversimplified though it is, helps to explain why denatured proteins are more readily split by proteolytic enzymes and why some native proteins are split so little.

There are well-known ways to separate the parallel strings in a protein, that is, to denature it. Among these, two might interest us now. One is to dissolve the protein in a strong solution of urea, or a similar substance (Kunitz and Northrop 1935; Eisenberg and Schwert 1951). The other is to heat it in the presence of water (Northrop *et al.* 1948). This latter situation is, of course, exactly what happens when a food product is pasteurized or blanched or sterilized by heating.

The foregoing diagrams should also make it evident, that the more completely these parallel chains of peptides have been separated, the harder it would be for them ever to come together again in their original relationship. If they did come together exactly as they existed before, they would form the original native protein again. Now suppose that the separation has not been quite complete—that the two strands still remain attached by a few remaining bonds that were stronger than the others. The protein might, if the threads didn't get hopelessly tangled, come back together and thus revert to its original form after the denaturing agent had been removed. This does happen with many proteins. In many cases if the strong urea solution is diluted greatly with water, or if the heated protein is cooled again before too much damage is done, the protein again resumes its native state, and we are right back where we started. If the protein solution is too concentrated, however, we get tangling. So it is apt to be a delicate operation, and it is quite easy to overdo the denaturing process. Changes in concentration, the timing, the temperature, and, above all, in the pH are apt to influence the results.

If this oversimplified picture seems fairly straightforward and satisfactory, let me now complicate it by introducing a proteolytic enzyme. It is to be remembered that the enzymes whose action we are discussing are themselves proteins—and in the case of the protein-hydrolyzing enzymes they appear to be rather simple proteins. So that all that has been said about proteins in general applies to the proteinases as well as to the proteins they are supposed to act upon. They undergo denaturation just as do their substrates. Also under some conditions they undergo reversion to the native state, which in this event means that they again become active as enzymes; for a denatured enzyme protein is never an active enzyme.

Hence, you may observe that the job of a proteinase in a food product is difficult. Just when the product is heated and the proteins in general become denatured, and thus become good substrates, the enzyme itself also becomes denatured and thus inactive.

On the other hand, if conditions are such that reversion to the native state is possible, the good substrates—denatured proteins—are apt to revert to their native forms, which are poor substrates at the same time that the denatured enzyme is recovering its activity. The effects seem to be a matter of timing: not all proteins are equally

denatured at the same temperature, nor do they all recover their native state at the same rate. Thus, a proteinase may be able to do a little business on the way down and again on the way back. For example, on the way down, papain is unusually heat-resistant. It is extraordinarily active in splitting other proteins which get denatured first during the heating period. On the way back, trypsin and chymotrypsin rapidly recover their native state (Balls and Jansen 1952) after heating, and find lots of work to be done on proteins that revert more slowly.

Another point in the behavior of enzymes towards proteins is that in no case is a single proteinase able to take a protein apart completely or even fairly completely to amino acids (Balls and Jansen 1952). A sort of hard core of resistant material the size of a large polypeptide is apt to remain. This may be easily attacked by some other proteinase, but it seems to be quite resistant to the enzyme of the first attack. It is not for nothing that our digestive system contains three distinct proteinases as well as a variety of peptolytic enzymes.

THE PROTEINASES

There is more to be said about proteinases than that they are simple proteins and may be divided into groups. All the books say that proteinases are enzymes that hydrolyze proteins. This is the truth. But it is not the whole truth by any means, if we are to judge by those proteinases whose action has been studied in any considerable detail. They do other things besides split proteins.

It is not surprising that proteinases also split small peptides since their amino acids are also linked together by peptide bonds. Similarly the amides of certain amino acids are also hydrolyzed, and release ammonia. These effects are very helpful in determining the type of amino acids in a protein that the enzyme prefers to attack, since such substances can be synthesized to order in the laboratory. However (aside from a few exceptional cases), a true proteinase acts very slowly on such substrates compared with its speed in splitting true proteins.

What is more surprising is the high speed at which proteinases split the esters of some particular amino acids whose side-chains seem to offer special attraction. In some cases the rate is about as fast as that observed for the splitting of peptide bonds in proteins.

Such enzymes are very efficient esterases for special types of esters. Trypsin, papain, and chymotrypsin would qualify as top-notch esterases if they had not been so well-known as proteinases long before their esterolytic powers were observed (Fig. 31).

The splitting of esters by proteinases has led to the finding of some other interesting reactions. For example, chymotrypsin splits *p*-nitrophenyl acetate, though not very rapidly. However, in the presence of a primary alcohol—glucose would do—the speed of reaction is greatly increased. This is only seen if one observes the release of nitrophenol from the ester, for very little free acetic acid is formed.

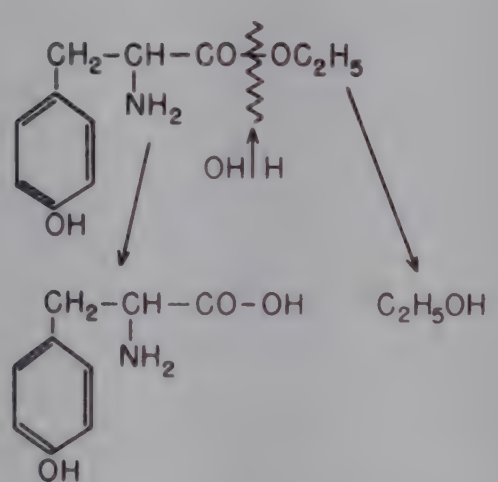


FIG. 31. TYROSINE ETHYL ESTER IS RAPIDLY SPLIT BY CHYMOTRYPSIN

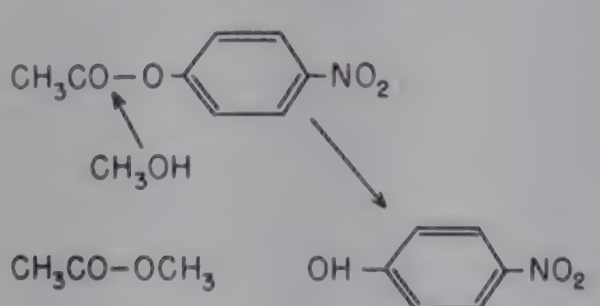


FIG. 32. THE FORMATION OF ETHYL ACETATE FROM NITROPHENYL ACETATE AND ETHANOL IS CATALYZED BY CHYMOTRYPSIN

4-glycyl-tyrosine amide
↓ cathepsin C
2-glycyl-tyrosyl-glycyl-tryosyl amide + $2NH_3$
↓
1-(glycyl-tyrosyl)$_3$-glycyl-tyrosyl amide + NH_3

FIG. 33. TRANSPEPTIDATION OBSERVED WITH CATHEPSIN C

What has become of the acetic acid? The enzyme has taken the acetyl group from the nitrophenyl ester and transferred it to the other primary alcohol group, thus forming a new ester at the expense of the old one (McDonald and Balls 1956; Fig. 32). This is a straightforward transfer reaction. It points to the possibility that transesterification, which is not necessarily related to protein-splitting, could occur in the presence of proteinases, for ester and alcohol groups also occur in natural products.

It may be objected that chymotrypsin is one of the digestive enzymes, and might be quite different in its action to the intracellular proteinases, which occur inside the tissues, although chymotrypsin does bear some resemblance to the choline esterases of nerve tissue and blood. Perhaps one should disregard these transfer reactions as not being typical of what may transpire in tissues themselves. However, this argument does not seem to be valid.

The intracellular proteinases, called cathepsins, have been extensively studied by Fruton and his coworkers. One of them in particular, usually obtained from spleen, but apparently rather widespread, has been found to catalyze transfer reactions involving small peptides. This *transpeptidation* can be demonstrated by permitting cathepsin *C* to act on a rather simple substance, 4-glycyltyrosine amide (Fig. 33).

The amide group is first split off and the peptide carried over to a second molecule of the substrate, where a new peptide linkage is formed and a new peptide. The process then repeats itself. In this way it was possible to build up an octapeptide, which is a pretty big peptide chain to be synthesized in the laboratory (Fruton *et al.* 1953). So not only can an intracellular proteinase catalyze a transfer reaction, but in doing so it can build up a sizable peptide chain using the energy that comes from splitting an amide bond.

The scope of proteinase action in tissues can thus be extended far beyond the original notion of simple protein hydrolysis. However, the synthesis of new substances by way of such transfer reactions as transesterification and transpeptidation must be considered. So the problem of the "effects of proteinases" becomes one of some complexity.

So far as food products are concerned, this problem would disappear at once if the enzymes in the tissue were permanently inactivated. That, of course, is one of the main objectives in food-process-

ing. But is it successfully attained? It is doubtful, particularly in the case of foods which have received what is termed the "high-short" heat treatment. Such treatment seems to me to offer the best chance for reactivation to occur if it is going to take place at all.

A good many years ago Schwimmer (1944) in my laboratory at Albany, California, broke down the enzyme peroxidase into two parts by heating it in aqueous solution. One part was denatured protein, which precipitated and could be spun off in a centrifuge. The other part was the well-known hemin group that had originally been attached to the protein and which remained in solution. When the solution and precipitate were mixed together under proper conditions the protein reverted, recombined with the hemin group and thus regenerated active peroxidase. It was of interest to do this with peroxidase from two vegtables, horseradish and turnip. These two could be distinguished from each other. So we put the turnip precipitate with the horseradish supernatant liquid and *vice versa*, to see what would happen. We regenerated peroxidase in all the mixtures; whenever we used turnip protein we got turnip peroxidase back, no matter what was the source of the soluble component. Similarly, we always got back active horseradish enzyme from horseradish protein. Since then other cases of reactivation have been observed. One recent example is the reactivation of phosphatase in pasteurized milk (Fram 1957). A test for active phosphatase has been used rather extensively to show whether milk has been properly pasteurized. Now it appears that milk that had been heated until the phosphatase was inactivated could redevelop active phosphatase under certain conditions, particularly if kept at room temperature. This may not be very serious, but it is sure to be a nuisance.

Just recently in my laboratory at Purdue University a very old experiment was repeated; chymotrypsin was inactivated by boiling it for about 10 minutes, then allowed to revert when the solution was cooled (Northrop *et al.* 1948). If the solution was acid enough and the amount of enzyme was not very large, complete reactivation occurred. Another way to do this is to dissolve the protein in 9 *M* urea and then dilute it with water about ten-fold. Nearly all the activity returns (Aldrich and Balls 1958).

An attempt was made to get this protein out of the urea solution without reactivating it. It was found that if the urea solution was diluted with 1 *M* sodium sulfate, instead of with water, all the pro-

tein was precipitated. The product was a perfectly inactive heavy white protein, quite insoluble in water. The sulfate was washed out with water; the protein allowed to stand first in alcohol, then in ether, and finally dried. (Brecher and Balls 1959).

Although this material was entirely inactive, it could be easily reactivated. This was done by redissolving the protein in strong urea solution, and then diluting the solution with water; just as though the protein had been active enzyme instead of an already denatured and, in fact, quite mistreated protein.

One curious fact about the reactivation is that the urea solution must be strong enough to have denatured and inactivated the active

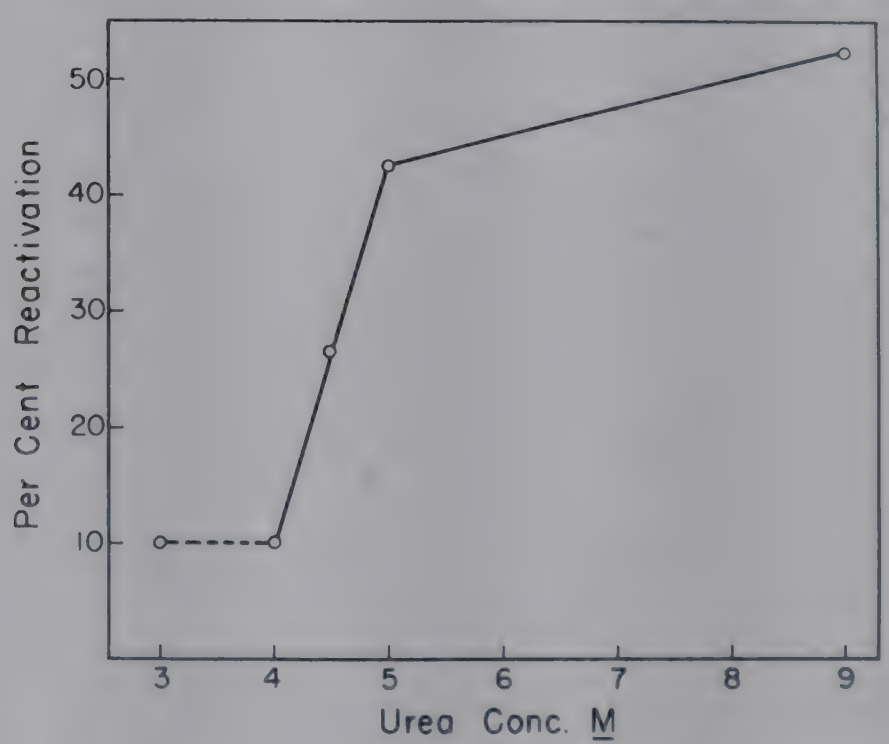


FIG. 34. THE REACTIVATION OF DENATURED CHYMOTRYPSIN REQUIRES A CONCENTRATION OF UREA HIGH ENOUGH TO DENATURE THE ORIGINAL PROTEIN

enzyme. If it is not strong enough to denature the active enzyme, it is unable to reactivate the already inactivated enzyme (Fig. 34).

Although this was not exactly gentle treatment for the enzyme protein whose activity it was hoped to recover, it had to put up with still worse. Such treatment was to dissolve the enzyme in urea, make the solution very acid (pH 2–3), then dilute it with ten volumes of alcohol. The solution was kept at room temperature for several days, then the protein was precipitated by adding a drop of sulfuric

acid. Sulfuric acid forms some sort of insoluble complex with chymotrypsin under these conditions. So does pyrophosphoric acid. The precipitate was then washed with alcohol and dried like the precipitates made with sodium sulfate.

These precipitates also regained their activity (in some cases almost all of it) when they were redissolved in urea and again diluted with water as before.

The important point here is that the reversion of a denatured enzyme protein to its active native state can take place much more readily than has been supposed.

This may not be important to the food industry. As far as is known, such things have been merely one form of sport in the theoretical laboratories.

SUMMARY

To sum up the highlights:

Proteins undergo a change called denaturation. While denatured they are much more susceptible to attack by protein-splitting enzymes. But these enzymes are also protein, also undergo denaturation, and are completely inactive while they are denatured.

Denatured enzymes, like other denatured proteins, often revert to the native (enzymatically active) state. The total effect during heating or cooling is one of timing. The enzyme that stays undenatured the longest can digest less heat-resistant proteins in the meantime. The enzyme that reverts the fastest can break down the proteins that are slower to revert, including other enzyme proteins.

The net result is complicated by the fact that proteinases split some peptides, amides, and esters as well as proteins. They can also transfer acyl groups or small peptide groups from one molecule to another and thus build up new substances by the transfers. The total of all these possible changes has never been properly studied and is certainly difficult to evaluate.

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B. S. Schweigert

Food Aspects of Enzymes Affecting Proteins

The impact of enzymes acting on food proteins is extremely broad, but is particularly evident in the case of the catheptic enzymes of beef muscle. In the present paper the theoretical knowledge on the action of purified proteolytic enzymes as applied to foods will be discussed. Trends in the types of methods used for studying proteolytic enzyme action in foods and the use of fractionation techniques for the preparation of purified catheptic enzymes carried out in our laboratory will also be considered.

SIGNIFICANCE OF PROTEOLYTIC ENZYME ACTION IN FOODS

The role of digestive enzymes in the breakdown of food proteins prior to absorption and assimilation is well known. Other than in diseased states involving a deficiency in the production of these enzymes or of hydrochloric acid in the stomach, the action of these enzymes is not the limiting factor in the "digestibility" of food proteins by man or other animals.

Much interest has developed in the influence of heat treatment (or the lack of it in the case of the trypsin inhibitor in legume seeds) on the "digestibility" or "availability" of amino acids from various foods. Attempts have been made to develop *in vitro* enzyme digestibility tests to reflect the suitability of thermal processes used in the preparation of foods and feeds. While a number of these have some promise, it is still necessary to conduct feeding tests to ascertain the protein quality of foods or feeds subjected to various processing treatments. Some success has been achieved on the application of "pepsin digestibility" tests to animal by-product feeds (Gehrt *et al.* 1955; Elmslie 1958).

It is of interest that studies in our laboratories have shown that the utilization of lysine from foods to support weight gains of rats is reduced by extensive heat treatment while tryptophan utilization is essentially unaffected. These and other tests have shown that part of the reduced utilization of lysine is attributable to decreased libera-

tion of lysine by proteolytic enzymes in the digestive tract when the foods have received a severe heat treatment (Guthneck *et al.* 1953).

The role of proteolytic enzymes added to foods in the process of manufacture or preparation may be illustrated by four examples:

1. Action of the enzyme, rennin, in the coagulation of milk proteins in cheese manufacture.
2. Use of papain or other enzyme preparations in the "chill proofing" of beer. In this instance hydrolysis of proteins in the beer prevents the development of undesirable colloidal suspensions of protein on chilling of the beer.
3. Use of micro-organisms as a source of proteolytic enzymes in the ripening of special cheeses (i.e., Liederkranz, Limburger, Camembert, and aged Cheddar). The microbial culture, for example, *Penicillium camemberti* in the case of Camembert cheese, functions as a source of extra cellular proteinase which slowly hydrolyses the proteins to give a smooth buttery texture extending from the exterior to the center of the cheese.
4. Use of proteolytic enzyme preparations (papain, bromelin, microbial proteases, etc.) for tenderizing fresh meats. In this case proteolytic enzymes, added in the form of powders or by dipping the meat in solutions of the enzyme preparations, act on the muscle fibers and/or connective tissue components during the early phases of the cooking process to increase the tenderness of meat (applicable primarily to beef steaks). The general aspects of the use of these enzymes have been reviewed recently (Bavisotto 1958).

ACTION OF PROTEOLYTIC ENZYMES IN THE TENDERIZATION OF MEAT

The effects of tenderizing agents will be limited to the role of proteolytic enzyme preparations in the present discussion. A complicating factor in studies of this type is that the penetration of the enzymes into the meat is limited, particularly when the preparations are added in powder form. In our studies this problem has been minimized by rehydrating frozen dried steaks in aqueous solutions containing various tenderizing agents. The action of proteolytic enzyme preparations has been followed by histological evaluations of changes that occur in various muscle and connective tissue components and by a trained taste panel evaluation of the initial tenderness and residue scores for control and treated steaks (Wang *et al.* 1958). A close relationship has been observed in the changes that occur in tissue structure and the taste panel scores. Manyfold higher concentrations of the enzyme preparations are needed to show histological changes than for differences to be detected by the taste panel.

In studies by Wang and associates (1958) relative potencies of enzyme preparations measured by hemoglobin and gelatin assay (Table 11) did not correlate in all cases with the histological and organoleptic findings. The data on hemoglobin and gelatin assays were kindly provided by Dr. Vincent Bavisotto of the Paul Lewis Laboratories.

TABLE 11

ACTIVITY OF PROTEOLYTIC ENZYME PREPARATIONS AS MEASURED BY HEMOGLOBIN AND GELATIN ASSAY[1]

Enzyme	Hemoglobin Assay		Gelatin Assay	
	Hemoglobin Units	Potency Relative Reference Standard	Gelatin Units	Potency Relative to Reference Standard
Reference Standard papain	5,300	1.0	1,300	1.0
Fungal amylase	1,900	0.4	1,500	1.2
Rhozyme P-11	2,900	0.6	6,700	5.4
Bromelin	14,800	2.8	18,200	14.5
Ficin	28,500	5.4	12,700	10.1
Papain	9,800	1.9	7,200	5.8

[1] From Wang *et al.* (1958).

TABLE 12

RELATIVE POTENCIES OF TWELVE ENZYME PREPARATIONS ON THE MUSCLE TISSUE COMPONENTS BASED ON STRUCTURAL MANIFESTATION[1]

Enzyme Preparations	pH	Muscle Fibers, Actomyosin	Connective Tissue Fibers	
			Collagen	Elastin
Protease 15	6.4	+++	−	−
Rhozyme P-11	6.8	++	−	−
Rhozyme A-4	7.3	++	−	−
HT Proteolytic	6.9	++++	trace	−
Fungal amylase	7.1	+++	trace	−
Hydralase D	7.4	+++	trace	−
Hydralast TP	6.9	++	trace	−
Ficin	5.2	+++	+++	++++
Papain	5.1	++	+	++
Bromelin	6.3	trace	+++	+
Trypsin	5.7	++	+	+
Viokase	5.8	+++[2]	+	+

[1] From Wang *et al.* (1957).
[2] Sarcolemma not affected.

Certain enzymes act primarily on the muscle fibers while others act on both the muscle fibers and connective tissue fibers as shown in Table 12 (Wang *et al.* 1957). It appears that only a small portion of the total tissue protein needs to be altered by proteolytic enzyme action, or else only a minor chemical change needs to occur in the

major proteins, to produce a significant change in tenderness as measured by a taste panel. On the other hand, relatively large changes are required to observe structural changes or to produce sufficient amounts of free amino acids for measurements of chemical changes. It would be most interesting to ascertain if the slight changes that presumably occur at levels for which differences can be observed by a taste panel could be observed by use of the ultracentrifuge or electrophoresis apparatus. Zender and associates (1958) have shown that the major effect of papain is to liberate soluble proteins from muscle while trypsin increases the quantities of amino acids liberated.

CATHEPTIC ENZYMES IN TISSUES

The aging of high quality beef cuts at temperatures just above freezing for 1 to 4 weeks has been commercial practice for many years. Experimentally, a comparable increase in tenderness can be achieved by aging for 24 hours at 110°F. with appropriate bacteriological control (Wilson *et al* 1959). The increase in tenderness observed, primarily during the first two weeks at 35°F. is thought to be attributable in a large part to the action of catheptic enzymes present in the muscle. An increase in autolysis can be observed microscopically, and increases in alpha-amino nitrogen and free amino acids are also noted. Muscle fiber extensibility decreases during aging (Wang *et al.* 1956). Representative data showing the changes in free amino

TABLE 13

EFFECT OF AGING OF MEATS UPON RELEASE OF AMINO ACIDS[1]

Sample	Amino N, Per Cent of Total N	Arginine,[2] Per Cent of Total	Tyrosine,[2] Per Cent of Total
Unaged Meat	0.8	1.3	0.24
Aged Meat	1.1	1.7	0.91

[1] From Ginger *et al.* 1954.
[2] Values determined microbiologically on the nonhydrolyzed NPN fraction.

TABLE 14

PROTEOLYTIC ACTIVITY OF VARIOUS ORGAN AND MUSCLE TISSUES

Tissue	Relative Activity
Kidney	1.9
Liver	1.5
Lung	1.0
Heart	0.33
Psoas Muscle	0.025

[1] From Zender *et al.* (1958).

acid nitrogen and certain amino acids during aging for two weeks at 35°F. are shown in Table 13 (Ginger *et al.* 1954).

It will be noted in this type of study that the muscle tissue is serving as a source of substrate as well as of enzymes at the normal pH of muscle after rigor (approximately 5.5). In studies by Doty and Wachter (1955) casein was used as a substrate and approximately 70 per cent of the proteinase activity of beef muscle was shown to be extractable by citrate buffer. The enzyme activity was estimated by measuring tyrosine liberation during incubation at pH 4.5 for 24 hours at 98.6°F. Approximately 50 per cent of the proteinase activity was lost after irradiation of the meat at a dosage of 1.6×10^6 rads, but no significant loss was detected at 0.5×10^6 rads.

The catheptic activity during storage of rabbit and lamb muscle obtained aseptically can be clearly demonstrated by liberation of amino acids, changes in electrophoretic behavior of the soluble proteins, and loss of striated patterns as observed by interference microscopy (Zender *et al.* 1958).

While the studies, by Doty and Wachter, referred to above, offered several advantages for the use of added substrate and measurement of the release of a specific amino acid, such as tyrosine, relatively little information was provided on the nature of the catheptic activity, i.e., number of enzyme components present, specificity, etc. Considerable work has been done on the catheptic enzymes present in liver, spleen and kidney, but relatively little with those in muscle, presumably because of the very low activity of muscle cathepsins as compared to the enzymes in organ tissues. In one study, for example, the presence of four catheptic enzymes in spleen has been detected by the use of synthetic substrates. A comparison of proteolytic activity for certain organ and muscle tissues has been made by Zender *et al.* (1958) and is shown in Table 14.

TABLE 15

ACTIVITY OF PROTEOLYTIC FRACTIONS OBTAINED DURING PURIFICATION[1]

System Studied	Tyrosine Liberated (μg.)	Specific Activity[2]	Purification Factor
Water extract of beef muscle	268	2.7	...
Ammonium sulfate fraction	440	10.0	3.7
Active fraction from DEAE cellulose column	640	48.8	18.2

[1] From Sliwinski *et al.* (1959).
[2] Tyrosine liberated per mg. of protein nitrogen per 4 hours.

In view of these facts, a research program was initiated in our laboratory to purify the cathepsin(s) of beef muscle (Sliwinski *et al.* 1959). In this study denatured hemoglobin was employed as a substrate at a temperature of incubation of 98.6°F. and an optimum pH of 4.4 was established. Balls (1938) described the proteinase activity of muscle and reported the optimum pH to be 4.1.

In our studies the proteolytic enzyme was purified 18-fold by the use of ammonium sulfate fractionation and chromatography on diethylaminoethanol-modified cellulose columns by the method of Mitz and Yanari (1956). The enzyme activity was shown to be proportional to the time of incubation for a six hour period with the appropriate choice of enzyme levels. Two major protein fractions were obtained by chromatography with the proteolytic activity present in only one fraction. As will be noted in Table 15, the specific activity of the fraction from the column possessing proteolytic activity was 18-fold over that observed for the water extract of beef muscle.

Subsequent investigations (Sliwinski *et al.* 1959A) have shown that the material possessing proteolytic activity obtained by chromatography is composed of several components which vary in their sensitivity to irradiation treatment. The use of synthetic substrates with each of the highly purified enzyme fractions is contemplated for the purpose of elucidating the specificity of action of the catheptic enzymes in beef muscle.

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The Control of Enzymatic Browning of Fruits

oncern the characteristics of enzyme reac-
as a basis for control of fruit browning dur-
early all of the oxidative browning of fruits
nol oxidase (Ponting and Joslyn 1948), the
ed to that enzyme.

ing to occur, three components must be
y, enzyme, substrate, and oxygen. If any of
prevented from reacting by some means, oxi-
ll not take place. This provides the basis for
ether the control is natural or artificial. We
y cases of natural control exist, as in canta-
h do not have either enzyme or substrate in
and in the unique Sunbeam peach, which
was deficient in substrate although the en-
this list could be added most of the berries
ever, a great many fruits and vegetables have
equired, and are ready and able to darken as
rganization is disrupted by bruising, cutting,
e the ones with which we are concerned in
general we do not want the effects of oxidative
e final products. There are some exceptions.
er the years has been difficult to keep from
become used to the brown color and accom-
rst do not always like a more natural color and
an example of this type of product. But in
are not fond of browned fruits, since it is diffi-
vned fruit from a rotten one, by either color or
less he knows otherwise, a processor usually

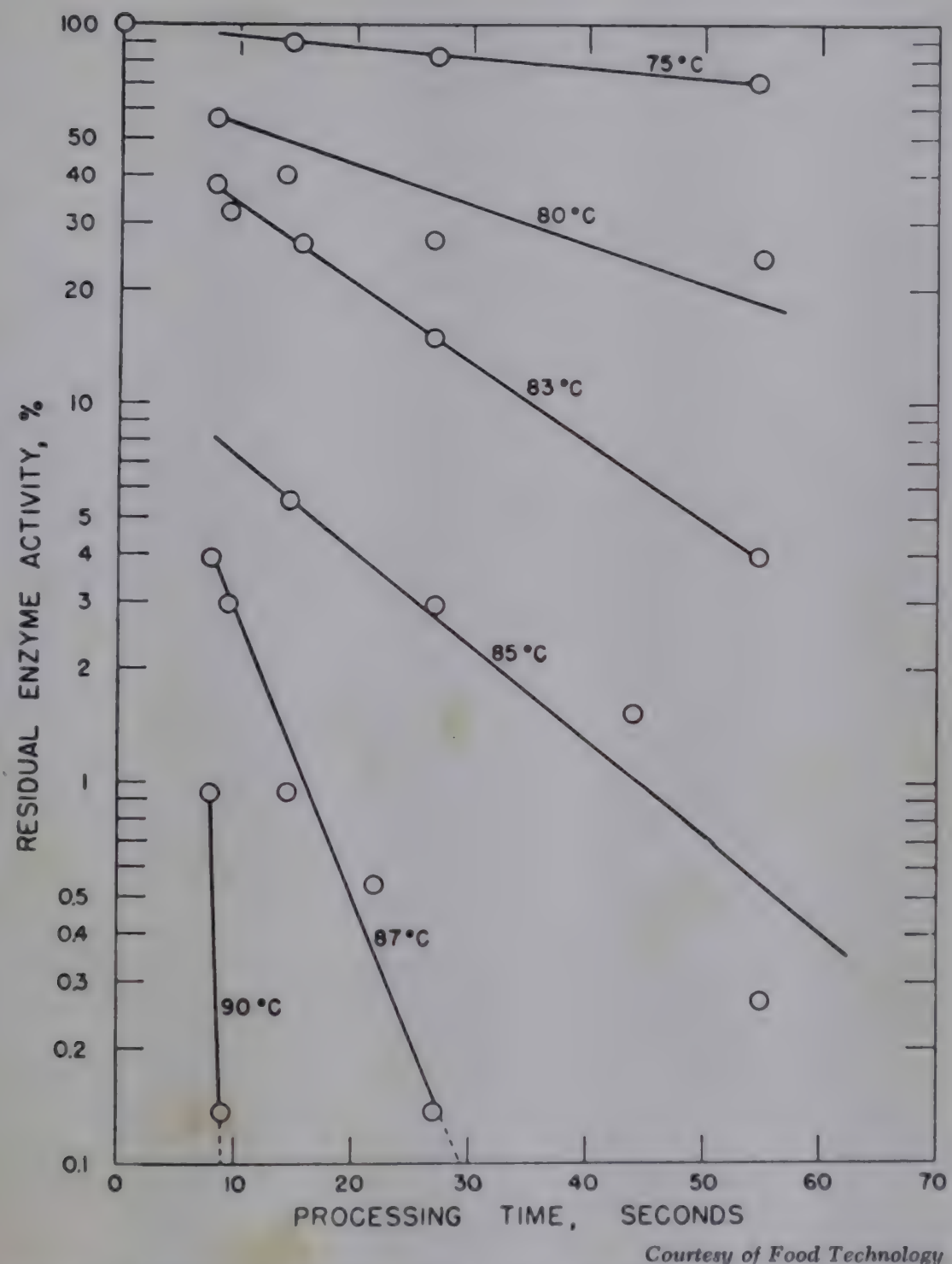
limited to inhibiting the enzyme or removal of oxygen, or sometimes a combination of these. Enzyme inhibition or inactivation and processes based on it will be considered first.

ENZYME INACTIVATION BY HEAT

Probably the simplest and most straightforward method of inactivating polyphenol oxidase, along with all other enzymes present, is to apply heat, since enzymes are proteins and are therefore easily denatured by heat. Heat is actually used commercially to a great extent in the processes of blanching or scalding, in canning, and in what is termed flash pasteurization. The trouble with heat is that it cooks the fruit and in some cases a cooked flavor is undesirable, even though it might not be a bad flavor. Also with some fruits, such as yellow passion fruit, there is very little flavor of any kind left after heating, although this fruit has a delicious and powerful flavor before heating.

Besides producing a cooked flavor, heat, if applied too long, softens the texture. This may be desirable in making purée, but undesirable in the packing of piece fruits, such as sliced frozen peaches. Consequently, blanching processes are usually a struggle to achieve suitable enzyme inactivation with a minimum number of undesirable changes in flavor and texture. The process must be specially tailored to each type of raw material and product desired.

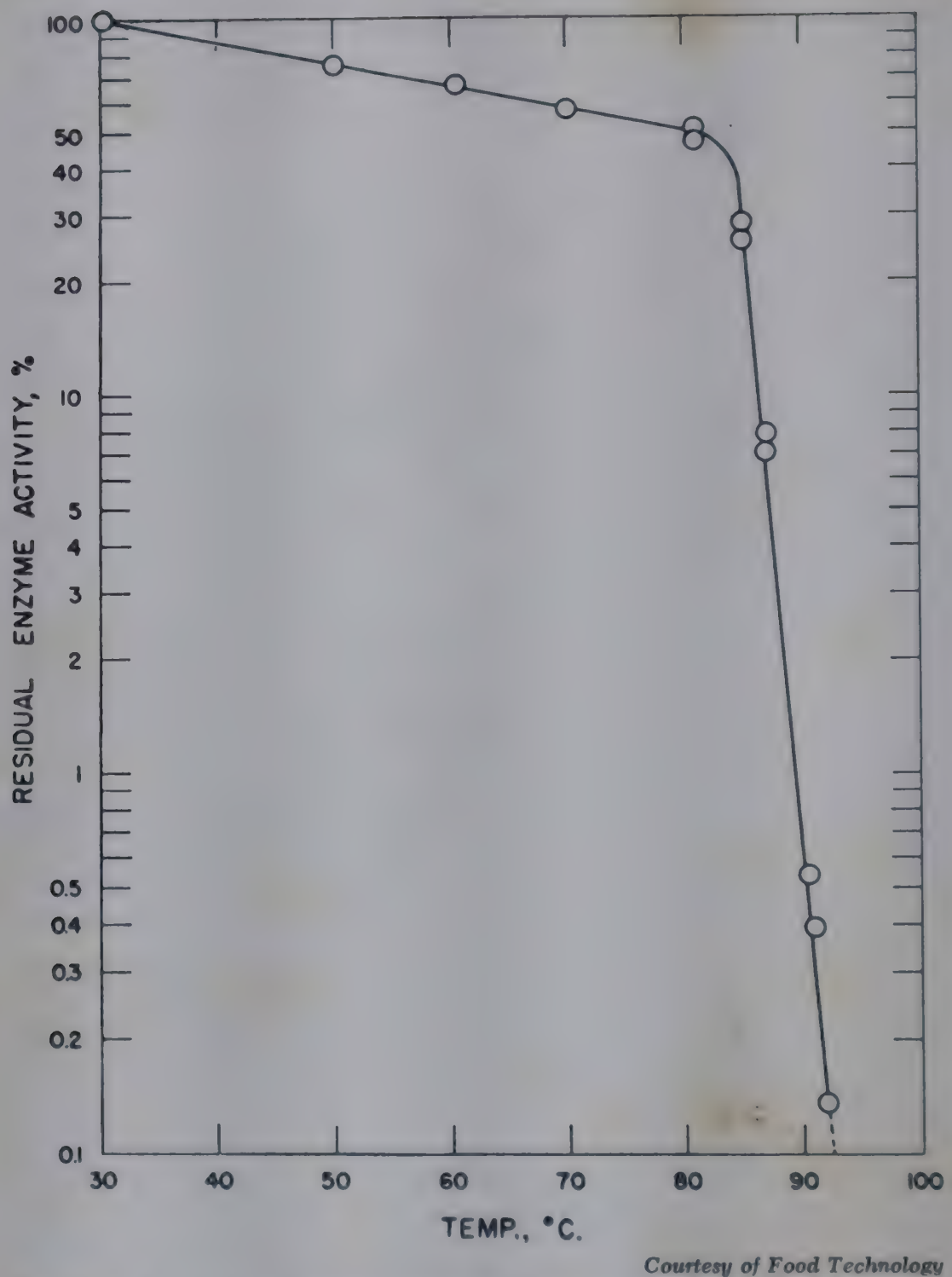
One common process we use for preparing fruit purée is to blanch the fruit whole or in large pieces in a screw-feed steam blancher, then pass the fruit through a paddle finisher, followed by a cooler. To be sure the fruit is heated only enough to inactivate the polyphenol oxidase, the purée is tested for enzyme activity with catechol as it comes out of the cooler, and the blanch time is regulated to be just sufficient for enzyme inactivation. This was found to give the best-flavored product in the case of apricot purée (Ponting *et al.* 1954). This type of purée, or the juice made from it, usually has a considerable amount of fresh fruit flavor. However, if fresh French prunes, for example, are made into purée and then into juice by this process, the skin color and acid will be extracted and the juice will be quite red and tart, somewhat like Italian prune juice. On the other hand, if the same prunes are pitted and puréed cold through a screened screw press, followed immediately by a flash heating and



Courtesy of Food Technology

FIG. 35. EFFECT OF HEATING TIME AND TEMPERATURE ON POLYPHENOL OXIDASE ACTIVITY IN PEAR PURÉE

cooling in a few seconds, the purée and the juice made from it will be yellow, very bland and sweet. In other words, it will have the characteristic color and flavor of the flesh of raw French prunes. This difference in character is due both to removal of a larger proportion of the skin in cold pulping and to less extraction of color and acid from the remaining skin because of the shorter heat treatment.



Courtesy of Food Technology

FIG. 36. EFFECT OF TEMPERATURE OF HEATING ON POLYPHENOL OXIDASE ACTIVITY IN PEAR PURÉE AT A CONSTANT (8 SEC.) HEATING TIME

A variation of the latter process has been used in our laboratory as the most satisfactory way to prepare juice from Thompson Seedless grapes. The cold grapes are puréed in a paddle finisher under a blanket of nitrogen, then immediately flash heated and cooled. After treating the purée with a pectin-destroying enzyme and pressing, the juice has the desired very pale color and fresh flavor.

In preparing apple segments for dehydrocanning, Powers and coworkers (1958) found that blanching the segments before drying resulted in an overcooked, mushy product. By drying the apples first, then blanching and filling hot, a much better product was achieved. Enzymatic browning was prevented during drying by keeping the air temperature low enough so that the internal temperature of the pieces was not over 120°F. (Walker *et al.* 1955). Above this temperature there was internal enzymatic browning. Surface browning was prevented by a dip in salt and citric acid solution plus the help of rapid surface desiccation, which in itself retards enzyme action.

A partial blanch was used by Guadagni and Nimmo (1957) for enzyme inhibition in sliced peaches before freezing. The peaches were packed in hot syrup, which inactivated the enzyme on the surface but did not soften the fruit excessively.

The effect of heat on rate of inactivation of polyphenol oxidase in fruit purées is illustrated in Figs. 35 and 36. Enzyme inactivation is very slow at 167°F., but becomes extremely rapid around 194°F. (Fig. 35). A sharp change in rate of inactivation occurs when a critical temperature is reached, in this case at about 180°F. (Fig. 36). The high temperature coefficient for inactivation above the critical point is similar to that of other protein denaturation reactions. At lower temperatures it is more nearly like that of ordinary chemical reactions. One point which should be emphasized is the short time required for enzyme inactivation above 194°F. A one-minute blanch of fluid fruit products near the boiling point may be 6 to 10 times as much as necessary. Overblanching to this extent is not often done at lower temperatures, where the time is longer, but it is fairly common at high temperatures. In other words, for best results the time must be controlled very closely at high temperatures.

A very nice commercial flash heating and cooling process has been described by Nury (1958) for inactivating enzymes and extracting skin color in viniferous grapes. The whole, stemmed grapes are pumped through a tubular heat-exchanger unit in which they are heated to 190° to 200°F. in about 25 seconds and then cooled back to 100°F. in approximately the same length of time. The processed grapes can then be made into juice without enzymatic darkening or heat damage which might occur during hot pressing.

CHEMICAL INHIBITORS AND ANTIOXIDANTS

Sulfur Dioxide

Many chemical compounds are effective inhibitors of polyphenol oxidase activity, but many of them are also toxic and cannot be used in food products. Of the chemicals used in foods, sulfur dioxide is undoubtedly the most widely used and probably also the most effective. There are some disadvantages to its use, however, such as its obnoxious odor during processing, the undesirable flavor it imparts to many fruits if used in excessive amounts, and the feeling by certain groups that it may be deleterious to health. On the other hand, it has been used for centuries with no obviously harmful effects; it is very inexpensive and so effective that its use is almost imperative in some products if they are to be edible.

Sulfur dioxide is a fascinating chemical because it is about as simple as a compound can be, yet it reacts with so many things that unraveling its chemistry in foods has been very difficult. It has been the subject of much research in the past, and could well continue to be so in the future.

The use of sulfur dioxide for inhibiting polyphenol oxidase in fruits has many aspects that should be considered. In piece fruits, the rate and extent of penetration is important. The amount of SO_2 reaching the center must be adequate for enzyme inactivation but not enough to cause off-flavor. Sulfur dioxide reacts with aldehydes and ketones, especially glucose, to form addition products which bind the SO_2 more or less tightly, depending on the type of aldehyde or ketone, the pH, etc. The relative effectiveness of free and bound SO_2 in inhibiting polyphenol oxidase has not been established, although several workers (Kerp 1904; Bioletti and Cruess 1912; Muller-Thurgau and Osterwalder 1914; Rahn and Conn 1944; Ingram 1948; Yang and Wiegand 1951) have found that in the analogous case of preventing fermentation of fruit juices only the free SO_2 is effective. The amount of free SO_2 depends on the concentration of SO_2, the concentration of glucose, and various other factors, many of which are unknown. It is peculiar that total SO_2, that is, the free plus combined SO_2, is almost always measured in fruits (for example, by the Monier-Williams distillation method), whereas the free SO_2 is alleged to be the only effective part.

A further complication found by Ponting and Johnson (1945), in

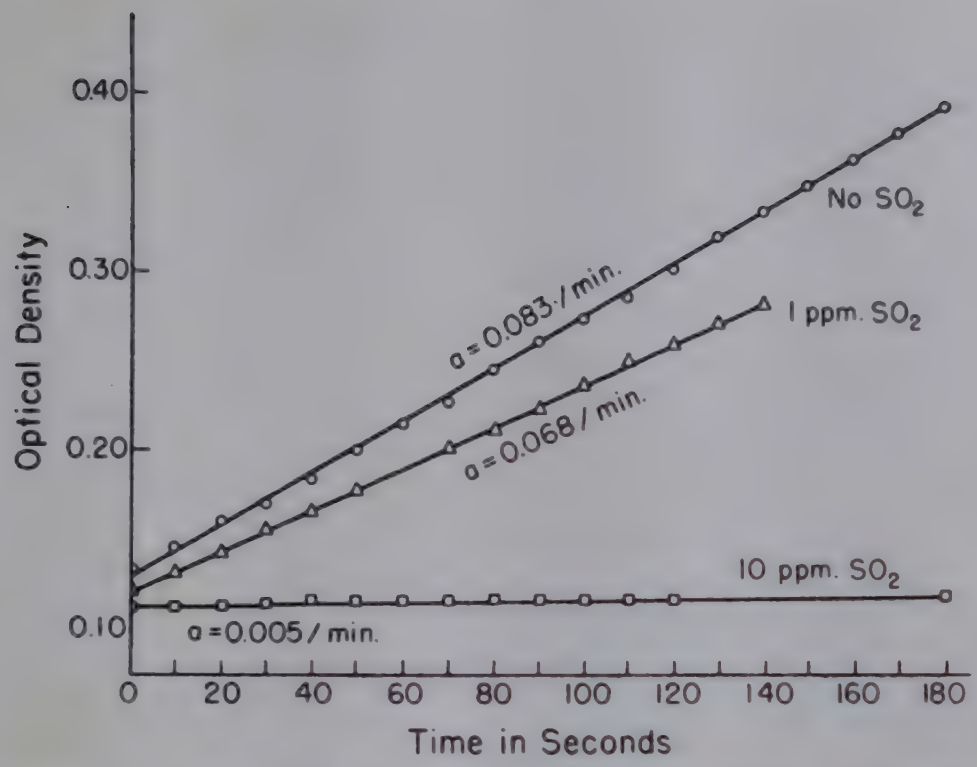


FIG. 37. EFFECT OF SULFUR DIOXIDE ON POLYPHENOL OXIDASE ACTIVITY

Sulfur dioxide added before catechol

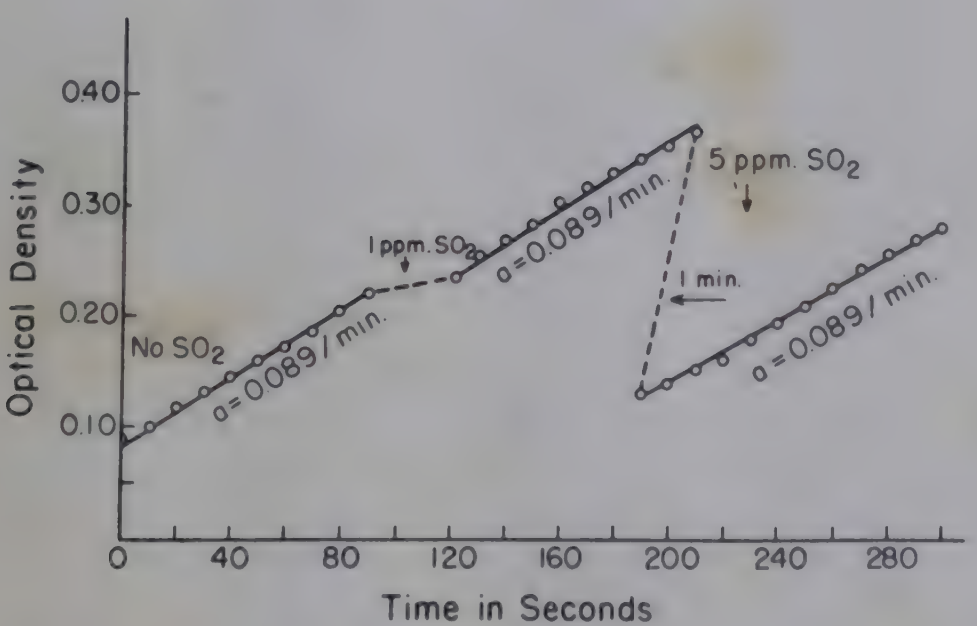


FIG. 38. EFFECT OF SULFUR DIOXIDE ON POLYPHENOL OXIDASE ACTIVITY IN APPLE JUICE CONTAINING CATECHOL

Sulfur dioxide added after some catechol oxidation. Curve moved back one minute on time scale, as indicated by arrow, for compactness

trying to measure SO_2 in the presence of active polyphenol oxidase in frozen fruits, was that part of the SO_2 was oxidized when the fruit was blended to extract SO_2. At the same time, the enzyme was inhibited. This raised the question of whether SO_2 used in the fruit was partially oxidized by this enzyme. Some recent experiments (Ponting 1959) have shown that this oxidation is due to the quinone formed by an enzymatic oxidation which had occurred before the SO_2 reached polyphenol oxidase. As a matter of fact, the enzyme is extremely sensitive to SO_2 when the two are brought together before any natural enzymatic oxidation occurs. Results of these experiments are shown in Figs. 37 and 38.

The rates of browning were measured (Fig. 37) when different concentrations of SO_2 (as $NaHSO_3$) were added to fixed amounts of buffered catechol solution. Equal amounts of a partially purified polyphenol oxidase preparation were added last. The slopes of the lines are a measure of enzyme activities, which are shown as the change in absorbancy or optical density per minute. Even 1 p.p.m. of SO_2 caused a significant drop in activity, actually about 20 per cent. Ten p.p.m. inactivated the enzyme almost completely. This is very nearly the same concentration that Downer (1943) found necessary to prevent growth of micro-organisms.

Fig. 38 shows the rate of browning in apple juice containing catechol, to which SO_2 was added after oxidation had proceeded long enough for some quinone to be formed. In this case the SO_2 reacts instantaneously with the quinone to lighten the color, as shown by the drop in optical density, but has no effect on the enzyme because the SO_2 has been oxidized before it can react with the enzyme. Even with 5 p.p.m. of SO_2 the activity is unchanged.

From these two experiments, it may be concluded that polyphenol oxidase is very sensitive to SO_2, but to be most effective, the SO_2 must react with the enzyme before any enzymatic oxidation occurs. This is analogous to the situation in preventing fermentation with SO_2. Downer (1943) found that although 10 p.p.m. of SO_2 would prevent fermentation, even large amounts added after fermentation had become vigorous would not stop fermentation. This was presumably due to the production of acetaldehyde which reacted with the added SO_2, as quinone did in the experiments cited above.

To go back to the problem of measuring SO_2, it appears that the oxidation of SO_2 found in blending frozen sulfured fruits was merely

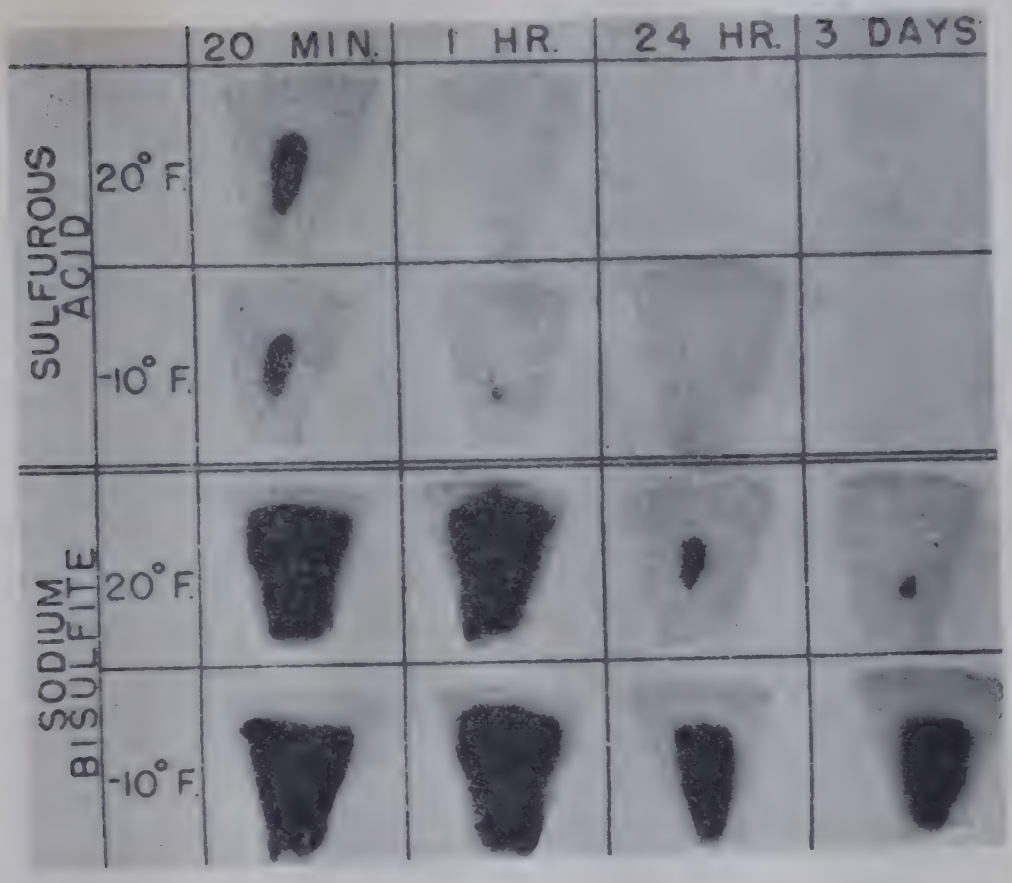


FIG. 39. CATECHOL TEST ON APPLE SEGMENTS TREATED WITH SULFUR DIOXIDE

due to improper mixing of the enzyme and SO_2 before freezing. In other words, the penetration of SO_2 into the fruit was incomplete. Consequently, when such fruit was blended, the natural substrate was oxidized enzymatically and the quinone that was formed oxidized some SO_2. This is somewhat different from the reaction in the presence of excess ascorbic acid in place of SO_2. With ascorbic acid the same type of indirect oxidation occurs but there is much less effective inhibition of the enzyme. In fact, as Ingraham (1956) has shown, there is no direct inhibition at all but only a gradual loss of activity as oxidation proceeds—the so-called reaction inactivation. Use of ascorbic acid will be discussed more later.

Penetration of SO_2 into fruits is important in preventing browning and this is especially true if the fruit is comminuted later, or if it contains much tissue oxygen. Apple segments, for example, contain enough oxygen in their tissue to cause internal browning if the enzyme is not inactivated. This browning may occur after thawing or even during frozen storage if the temperature is not very low; also

during drying in the production of dehydrocanned or dehydrofrozen apples. The rate of penetration of each molecular species of SO_2 is different, that is, it varies with pH, but the extent of penetration can be tested by spreading a one per cent solution of catechol across the newly exposed surface of a piece of fruit which has been cut in cross section. Where the enzyme is still active, the catechol will be oxidized and turn brown or black within a few minutes (Ponting 1944).

Fig. 39 shows the sharp contrast that can be achieved by the catechol test. Also, it shows the different rates of penetration of sulfurous acid and sodium bisulfite in apples. If sulfurous acid is used, the penetration is almost complete in 20 minutes even at 20°F., so that the apples can be frozen immediately. However, if the apples are dipped in sodium bisulfite, they must be held before freezing for about 24 hours at 20°F. before penetration of SO_2 is complete. At 60° to 70°F. the holding period would be about eight hours. The reason for using sodium bisulfite at all, or a mixture of this with sulfurous acid, is that there is very little SO_2 odor, and, therefore, working conditions are much more acceptable.

In many other fruits, such as apricots and peaches, there is not enough tissue oxygen to cause internal browning as in apples. Therefore, it is not necessary to penetrate the whole piece of fruit with SO_2. This is fortunate indeed, because even gaseous SO_2 does not penetrate these fruits very well. Powers and co-workers (1956) found that apricots for dehydrofreezing could be effectively treated by the following procedure: The whole fruit was dipped in a strong sulfurous acid bath, so that SO_2 penetrated just under the skin where most of the enzyme activity was located. Then the apricots were cut in half and dipped in a very weak SO_2 solution to protect the cut surfaces where the enzyme activity was low.

One more important item in the use of SO_2 is its removal from treated fruits. Since SO_2 is most effective when used in high concentration to inactivate the enzyme rapidly, it is often desirable to remove or react the excess after treatment to prevent off-flavor and -odor. If penetration has been good, SO_2 can be removed right away, since there is no evidence of regeneration of the enzyme. In preserve and piemaking, the SO_2 is removed fairly well by cooking, but in other products it may be easier to use a chemical that reacts with SO_2. Hydrogen peroxide is such a chemical, but its use may not be permitted in some foods.

Acids

After sulfur dioxide, the most widely used chemical inhibitors for control of enzymatic browning are acids. This group includes both inorganic and organic acids, as well as those with reducing power such as ascorbic acid. Polyphenol oxidase activity is directly dependent on pH, in addition to any special effect such as the reducing action of ascorbic acid. Where the effect of lowered pH is not undesirable in a fruit product, the use of an acid is probably as simple and effective a treatment as can be used. For example, after lye-peeling cling peaches, an acid rinse is very useful in preventing discoloration by lowering the pH which had been raised by residual lye. Also, acids are often incorporated into solutions in which fresh-cut fruit is held prior to processing.

Fig. 40 shows the effect of pH on enzymatic browning of apple juice. In this case, hydrochloric acid and sodium hydroxide were used to adjust the pH. Below pH 2.5 to 2.7, all enzymatic activity ceased. Furthermore, if the pH was subsequently raised to the original value, the enzyme did not recover and no browning occurred. This offers the possibility of enzyme control by a two-step acid treatment which will be discussed later.

The stability to heat of the enzyme increases with pH up to about seven; thus it bears little relation to the natural pH of the fruit. This is illustrated in Fig. 41, which shows the effect of pH on thermal stability of polyphenol oxidase in pear purée.

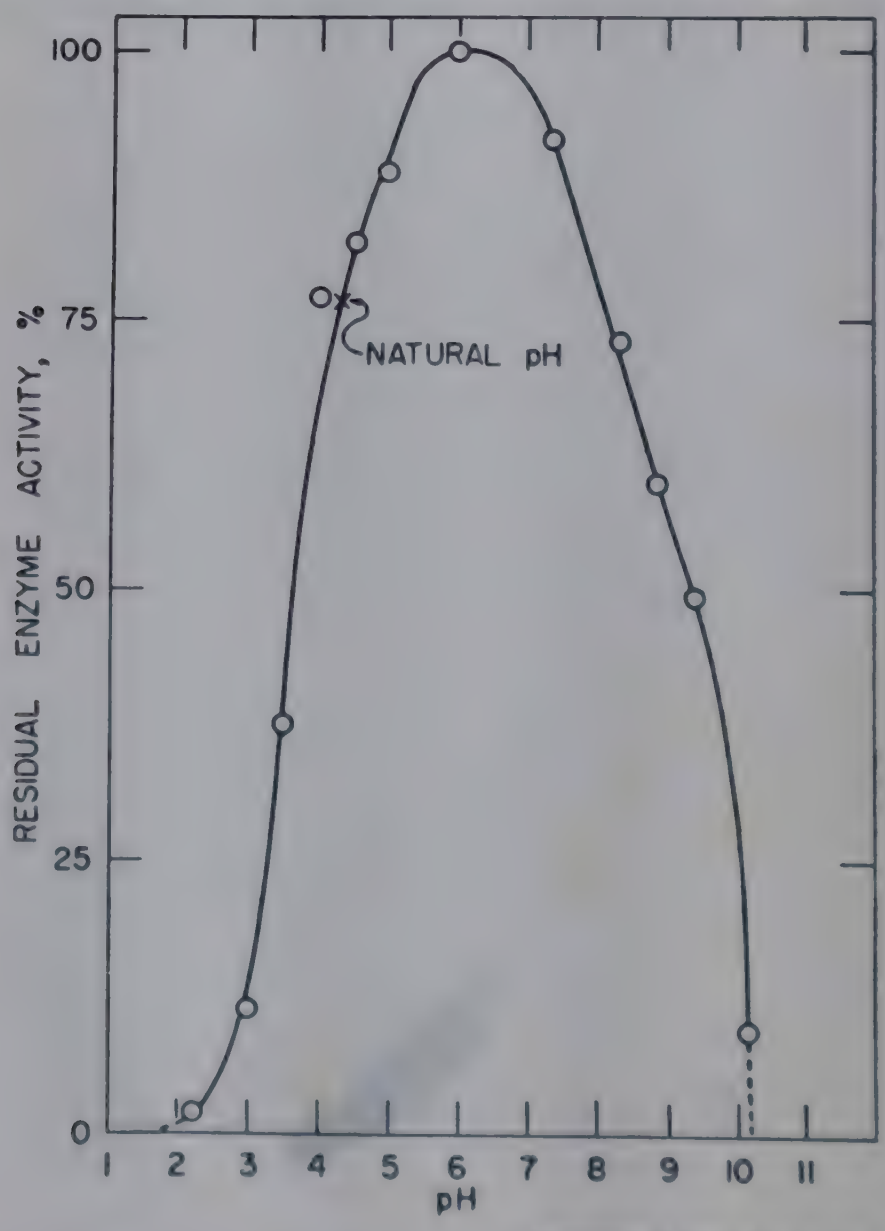
FIG. 40. EFFECT OF pH ON ENZYMIC BROWNING OF APPLE JUICE

pH values are shown below corresponding tubes

As can be seen from the curve in Fig. 41, a ripe fruit with a high pH would be more difficult to blanch than a less ripe one. Consequently, blanching times might have to be varied considerably over a season to relate to the pH of the fruit.

Besides the effect of pH alone, there is a great difference in the effects of specific acids on polyphenol oxidase activity in fruits. A comparison has been made of two concentrations each of malic and citric acids in apple juice (Fig. 42). It is apparent that malic acid is much more effective in preventing browning.

Since malic acid is also the main acid in apple juice, it could be used in a process to prevent enzymatic browning in apple juice. In



Courtesy of Food Technology

FIG. 41. EFFECT OF pH ON THERMAL STABILITY OF POLYPHENOL OXIDASE IN PEAR PURÉE

such a process (Fig. 43), malic acid is added to the apples as they are being ground for pressing. Otherwise, the juice is handled as usual. After the juice is cleared by filtration, it is run through ion exchange columns to recover malic acid, which can be used again on fresh apples. There might be a net gain of malic acid or a slight loss, depending on the pH of the fruit and the desired pH of final product. This pH would depend on sugar content. An extra dividend is the standardized pH that can be obtained regardless of the maturity of

FIG. 42. EFFECT OF MALIC AND CITRIC ACIDS ON BROWNING OF APPLE JUICE

Percentages of acids and pH values are shown below corresponding tubes

the apples. The flow of juice through the ion exchange column is regulated to remove the proper amount of malic acid and thus achieve the desired pH.

Although it is feasible to prepare a natural-colored apple juice by various processes, this kind of juice may not be as acceptable to consumers as a browner juice. However, the public may eventually appreciate the natural flavor and color of unbrowned juice. This is a situation similar to that in the brewing industry in Pilsen over a hundred years ago. The story is told that all beer at that time was quite dark, but once some brewer made a mistake and didn't heat the malt enough to brown it. The beer made with it turned out to

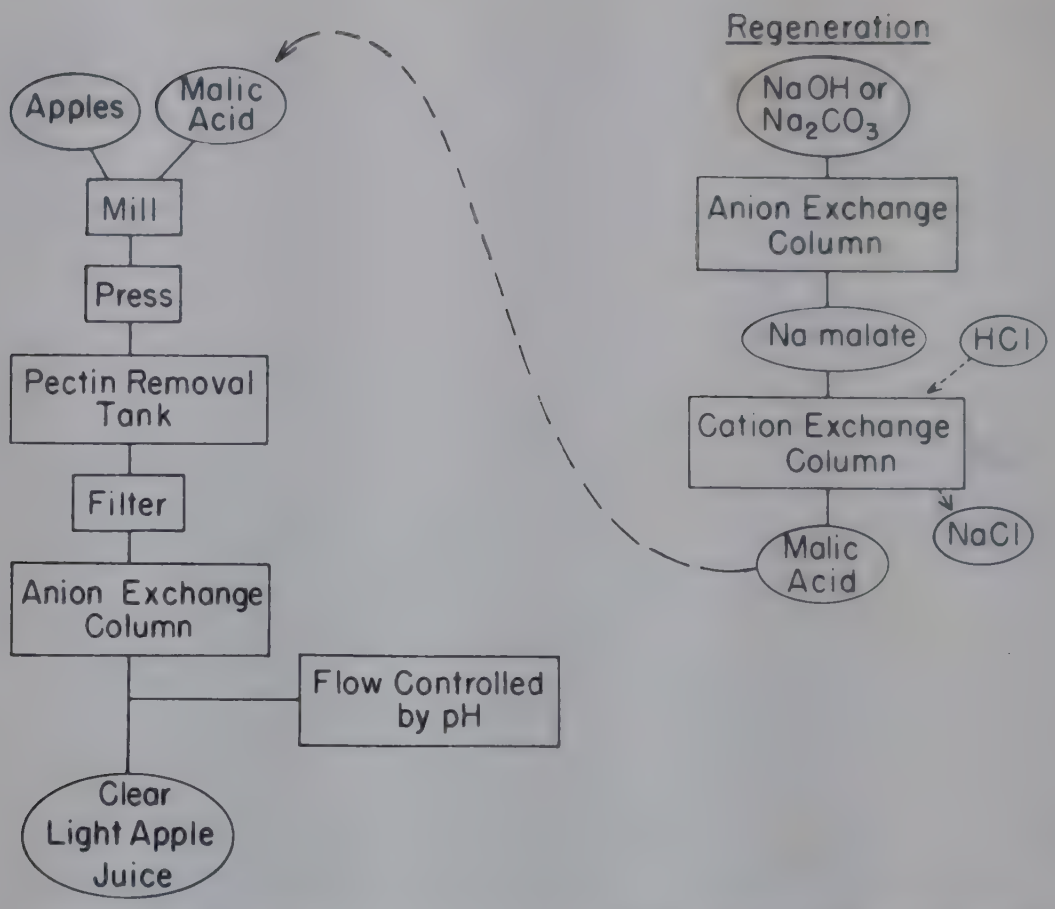


FIG. 43. FLOW SHEET ON USE OF MALIC ACID IN PROCESSING APPLE JUICE

be quite light in color, but the flavor was good, so they kept making it the new way and eventually Pilsen became famous for its light beer.

Ascorbic acid.—Ascorbic acid has been used a great deal to control enzymatic browning in fruits, probably more than any other acid. This is because it is a good reducing agent and prevents formation of brown oxidation products; it may also aid slightly in lowering pH. Rather than being a non-nutritional additive, it is valuable as a vitamin.

There have been many ways of using ascorbic acid in processing fruits, but not all of them have been soundly based. Ascorbic acid itself is not an inhibitor of polyphenol oxidase in the way that sulfur dioxide is. It must be oxidized indirectly by the enzyme before it can inhibit the enzyme activity. That is, if there is sufficient ascorbic acid present, polyphenol oxidase will oxidize its natural substrate and the oxidation product will be immediately reduced by ascorbic acid, this process being accompanied by a gradual decrease in enzyme activity until finally the enzyme is all inactivated. This is called

reaction inactivation, because it does not occur in the absence of an oxidation reaction. In the course of the inactivation, a considerable amount of ascorbic acid may be oxidized.

If an insufficient amount of ascorbic acid is added to prevent browning until the enzyme is inactivated, there will merely be a slight delay in browning until the ascorbic acid is gone, then it may proceed as usual. Therefore, it is almost useless to treat fruits that brown badly with a small amount of ascorbic acid. The effect is not at all proportionate to the amount added. However, if a fruit is

FIG. 44. APPLE JUICE PLUS ASCORBIC ACID ONE HOUR AFTER MIXING

Per cent ascorbic acid is shown below corresponding tube

treated with sufficient excess of ascorbic acid to allow the enzyme to inactivate itself, browning can be prevented effectively and permanently. Of course, this applies mostly to fluid products because of the problem of penetration into piece fruits. It is often very difficult to prevent browning in piece fruits by applying ascorbic acid to the surface, because the active enzyme in the center of the piece can continue to oxidize the ascorbic acid as it penetrates. Nevertheless, in freezing apricot halves, for example, a fairly high concentration of ascorbic acid applied to the surface just before fast freezing might prevent browning upon thawing, while a lower concentration or even the same amount applied an hour or two before freezing might show no effect at all.

FIG. 45. APPLE JUICE PLUS ASCORBIC ACID FOUR HOURS AFTER MIXING

Per cent ascorbic acid is shown below corresponding tube

Figs. 44 and 45 demonstrate the effectiveness of ascorbic acid in maintaining the natural color of apple juice. Incidentally, apple juice was used for convenience; other fruit juices undergoing enzymatic oxidation would serve equally well. Juice treated with one per cent ascorbic acid remains perfectly unbrowned, whereas even with as much as 0.5 per cent, browning is not controlled (Fig. 44). Much of the activity has been destroyed, but there is enough left to cause slow browning. One-tenth of one per cent ascorbic acid is practically worthless, yet many a processor has used this concentration.

Fig. 45 shows the same tubes after four hours at room temperature. The tube containing one per cent ascorbic acid is unchanged and will remain so, but the others show a ring of browning around the top where oxygen is present. In deep tubes like these, the juices are deoxygenated by ascorbic acid oxidation.

Other Chemical Inhibitors

Of the many salts which act as inhibitors of polyphenol oxidase, only sodium chloride is used extensively in food, and inhibition of polyphenol oxidase by this salt is often incidental to prevention of spoilage by microorganisms. However, for short periods of holding

peeled fruits for processing it is very useful. Many combinations of salt with ascorbic acid, citric acid, etc., have been used for this purpose. However, when the enzyme must be inactivated more completely, the concentration of salt required is too high to be palatable. To inhibit enzymatic oxidation during the determination of SO_2 Ponting and Johnson (1945) found that a 20 per cent concentration of salt was required.

A chemical inhibitor which could supplant sulfur dioxide would be highly desirable in view of the drawbacks to SO_2 mentioned earlier. Not much progress has been made along this line. Jansen (1958a, 1958b) has obtained two patents on sulfhydryl compounds for use as inhibitors of plant enzymes. One of these patents (1958b) is on compounds of the formula HS-R-X, where R is an aliphatic radical of at least 3 carbon atoms and X is OH or SH. The other patent (1958a) is on N-acyl derivatives of mercaptoamines, of the formula R–CO–NH–R′–SH, where R′ is an aliphatic hydrocarbon radical. These sulfhydryl inhibitors are very effective but have an undesirable odor. Other sulfhydryl-containing substances such as cysteine also are usually effective against browning but have undesirable tastes or odors. There is so far no odorless, tasteless, non-toxic, and cheap enzyme inhibitor with the effectiveness of sulfur dioxide in sight.

REMOVAL OF OXYGEN

Oxygen removal is the most satisfactory way to maintain certain types of fruit products in the most nearly natural state, especially where texture and flavor are very important. For instance, in frozen sliced peaches the polyphenol oxidase is not ordinarily inactivated in the interior of the slices, but by treating the surface of the slices with excess ascorbic acid before freezing, oxygen on the surface can be effectively removed. The ascorbic acid maintains a barrier to diffusion of oxygen into the fruit from the headspace of the package, and the diffusion of oxygen into the headspace from outside is retarded by the package and the low temperature. Of course there may be weak links in the chain, as pointed out by Guadagni and Nimmo (1957), and browning can occur if the temperature is not low enough, if the package is too porous to air, or if the ascorbic acid is insufficient in quantity. Nevertheless, it is not too difficult to maintain all the proper conditions and obtain an excellent product. Or, instead of using ascorbic acid, the peaches can be packed under

vacuum in a hermetically sealed can to prevent entrance of oxygen.

A product similar to frozen sliced peaches is halved apricots frozen in 30-lb. friction-top cans. Since there is little tissue oxygen in the apricots, the fruit browns only at the top surface of the material in the can. By applying a little ascorbic acid or sulfur dioxide to the surface, browning is prevented with a very minimum of treatment. Even sugar or syrup is of some use as a surface cover but it only retards diffusion of oxygen and is not effective as an antioxidant.

Apples cannot simply be packed into cans and treated on the surface, because of the tissue oxygen they contain. One way that has been used to remove their tissue oxygen is to let the apple slices respire under water or brine until they use up all the oxygen (Clark 1923). This requires 20 to 30 minutes at 120° to 130°F. However, Powers *et al.* (1958) in dehydrocanning apples found this to be a somewhat ineffective system. Better results were obtained by drying at a low piece-temperature followed by blanching and filling, as mentioned earlier.

An alternative procedure to respiration of apple slices is vacuum-treating in a water or syrup bath to remove air from the fruit and permit liquid to replace it when the vacuum is released (Sellars 1918; Grab and Haynes 1948; Guadagni 1949).

If fruits are pulped, deaeration alone usually cannot be used to prevent browning because the enzymatic reaction is too fast. If the fruit contains a large amount of natural ascorbic acid the deaeration might be achieved before browning starts, but ordinarily an inhibitor must be added or the fruit blanched to maintain the natural color. To exclude oxygen momentarily during pulping, a blanket of inert gas can be used, as described above for preparation of light-colored grape juice.

A good type of machine to have for processing purees would be one in which the fruit is milled and flash-pasteurized in the same unit, in a few seconds. This does not seem to be impossible to design, and perhaps such equipment will be available in the future.

SUMMARY

Control of enzymatic oxidative browning of fruits catalyzed by polyphenol oxidase is based on the three reactants in the oxidation, namely, the enzyme, its substrate, and oxygen. Most control processes depend on partial or total inactivation of the enzyme, either by

heat or by chemical inhibitors and/or antioxidants, such as sulfur dioxide and ascorbic acid. Oxygen removal and exclusion is also useful in some products, especially when combined with at least a partial enzyme-inhibition treatment. The substrate cannot be readily controlled but occasionally a low-substrate variety of fruit can be selected.

In general, the best processes for control of enzymatic browning are those in which the enzyme is inactivated very rapidly, before any appreciable oxidation has occurred. This applies to both heat and chemical treatments. Such processes tend to result in the least alteration in natural color and flavor.

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E. M. Mrak

Summary of the Symposium

This has been an inspirational meeting with magnificent papers covering a wide range of subjects. The Department of Food and Dairy Technology of Oregon State College is to be congratulated for sponsoring a symposium that emphasizes the importance of enzymes in the broad field of food technology.

Though this has been termed a symposium, according to Webster's International Dictionary, the second edition, 1957, it is not in the true sense a symposium. According to Webster, a symposium was "In ancient Greece, a drinking together; a compotation, usually following a banquet proper, with music, singing, and conversation; hence a banquet or social gathering at which there is a free interchange of ideas. At such gatherings there might be a discussion on such a subject as Plato reporting on ideal love." Webster also indicates that a symposium might refer to "a conference at which a particular topic is discussed with various opinions gathered; also a collection of opinions on a subject." At this meeting we have had a collection of opinions on a very broad and important subject.

During the opening of the symposium, Professor Wiegand covered historical aspects of food science; Dr. Paul Cannon discussed the work of the National Institutes of Health Study Section on Toxicology, which helped sponsor this program. He pointed out the need for research in the area of toxicology and the interests of the committee in promoting research in this field.

The magnificent and inspiring paper by Dr. Green should be available to every young scientist, as it is an indication of the opportunities that lie ahead. It gave background for a multidisciplinary approach to further research in an important field. He indicated the need to consider all areas of science in developing a particular field. He emphasized the importance of considering, in the future, the relation of enzymes to biological structure. If we look upon organization as a chemical process, then once we find the key to the structure of the cell, we can make further great progress in enzymology. This means, in terms of research, that university scientists, including

geneticists, microbiologists, virologists, embryologists, and just general biologists will find it necessary to work on a team basis.

A discussion of enzymatic darkening added more to the cloth woven by Dr. Green, indicating that eventual understanding will be related to organization. In line with this, the question remains: Why doesn't the whole tissue darken rather than just certain elements?

The other papers each presenting important aspects concerned with mechanisms of enzyme activity relating to carbohydrates, lipids, or proteins, have at least indirectly brought out applications in the field of food technology.

The thread running through the entire conference indicated the importance of cell organization, of interdisciplinary work, of the need for cooperation of people in different disciplines, and above all, the importance of enzymes in the field of food science and technology. The need for food scientists to consider the very fundamental aspects of enzymology, to have an understanding of the basic

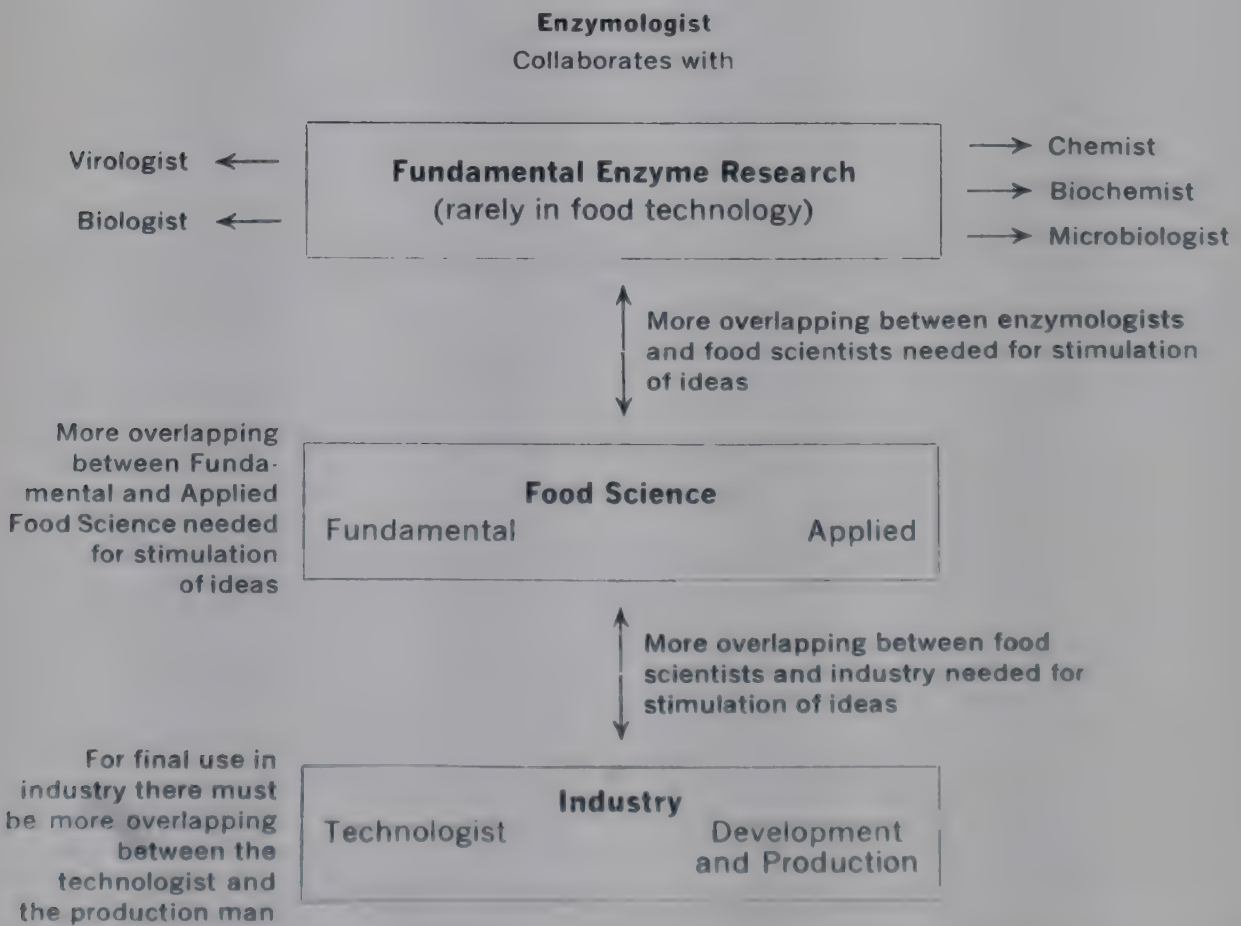

FIG. 46. THE POSITION OF FOOD SCIENCE BETWEEN FUNDAMENTAL ENZYME RESEARCH AND THE APPLICATION OF THE KNOWLEDGE OF ENZYMES

areas in this important field, and above all, to communicate with others working in the field, was clearly indicated. The field of food technology needs more than a thread; there must be a fabric where the woof involves the fundamental aspects and the warp involves the applications. Without such overlapping, there will be a genuine lack of stimulation, a lack of sound ideas and a great delay in applications.

The question remains as to how the various points of view, including fundamental research, applied research, and industrial utilization, may be brought together. It is apparent that great gaps remain between these three groups. This has been brought out very emphatically by the questions asked at this conference. While listening to these questions, I tried to conceive in my mind some method of obtaining sound communication and stimulation between these various groups, for if this is not done, the art in food technology will not yield to the science, and our advancements will be delayed, if not stopped. We would continue to grope, rather than proceed on a sound basis.

I have tried to indicate the great gaps between the various points of view diagrammatically (Fig. 46) and at the same time to show that, on the one hand, we have people working in fundamental areas and, at the other extreme, we have people in industry applying the results. In between, we have food science research which involves both fundamental and applied efforts.

This conference has made it clear that unless there are better communications between the various groups, and unless there is hybridization and stimulation, the ideas that result in application will be slow in coming. It is apparent that industry, food technologists in general and food scientists in universities must realize this at the departmental level. It has been made clear here that much teaching and research in food technology has lacked this type of stimulation and has not been properly balanced in that it has been directed largely to the applied and production point of view without the benefit of the fundamental point of view.

B. E. Proctor*

Perspectives in Food Research

It is especially fitting that special cognizance is being made at this meeting of Ernest H. Wiegand and his pioneer efforts which did so much to make possible both the outstanding place that the State of Oregon now occupies in the food production of our nation and the fine reputation of the Oregon State College and its graduates in the field of food technology and the food industries.

The early and diligent endeavors of Professor Wiegand, his colleagues, and his successors have made possible this active and dynamic center of training for many future well-trained men for the conversion of the agricultural products of Oregon into food products to feed the world. The status of the food industry and its important products and by-products in Oregon and the United States has been enhanced by the progress which has been made in this college by the research and teaching related to raising the quality and quantity of products and processes for the benefit of agriculture, the processors, and the consuming public.

One should not omit reference to any of the many important food products of Oregon. These, of course, include milk and milk products, the many fruits and vegetables, and the fisheries, which constitute sources of manufactured foods which are increasing in importance in national and international trade.

Food research itself is now recognized as a factor of primary importance to all nations, large and small, because the population increases which are taking place all over the world make food a factor of *existence* in some areas, while in others food technology may be intimately connected with dreams of domination.

THE FOOD INDUSTRY IN OTHER COUNTRIES

It may be of more than passing importance to know what is going on in the food industries of some other countries. With the encouragement of government, food production may be increased rapidly

* Deceased.

sometimes. During a recent two-year period, it is cited, the following new government food plants were built in Russia:

144 meat processing plants
50 canneries
744 dairy plants
372 bread factories (some are said to make 900 tons per 24 hours!)

Great efforts have been concentrated on the fisheries industry and many floating fish canneries and freezers have been launched during the last decade in Russia. Present plans are said to envisage seven billion pounds of processed fish a year in the near future using large freezing trawlers which currently freeze half of all the fish caught. Progress has also been made in modernizing the dairy industries, bakeries, fruit products and beverage industries, and in baby foods manufactured by the Soviets.

While all these improvements are being made, it is noted that the availability of meat there, in terms of pounds per person, is even now only a third of that which we enjoy in the United States. From a nutritional standpoint, it is also significant to indicate that in the Russian diet two-thirds of all the protein available for human foods is plant protein in contrast to our country where two-thirds of our dietary protein is animal protein, which is of higher quality.

CHANGES IN THE UNITED STATES

Coming back to the United States, there are some interesting comparisons to be made. In early colonial days, perhaps 85 per cent of our population worked to feed everyone. Today 15 per cent of our population feeds themselves and everyone else in this country. In recent years, we seem also to have had *plenty left over* to distribute in other countries. Some of these products have been raw materials such as grain; others have been fabricated products like butter, cheese, and dried milk.

It is significant that the development of quantity production in foods has inevitably been associated with *higher quality*, and the improved products have been accompanied by *increased demand!* Surely the place to note this is in our modern supermarkets which now carry some 6,000 different food items compared to 1,000 in 1930 and less than 500 in 1900.

EMANCIPATION OF THE HOUSEWIFE

The reason for this great revolution in the food industry stems back to the emancipation of the housewife from many of the responsibilities which chained her in earlier centuries to the kitchen stove, the sink, the pump, and the manual conversion of raw materials in the form of vegetables, fruits, milk, flour, chicken, veal, fish, and spices to the finished products of meats, cereals, butter, bread, and desserts.

Today and/or tomorrow she will enjoy electric or radar ranges in the wall at table height, which cook food in a small per cent of the time formerly required. Dishes will be ultrasonically washed in a few minutes if, indeed, dishes are not discarded because they will have become the food packages. The menus will require only an IBM symbol to be punched as each component pops out of the freezer to be heated and assembled into a complete dinner by automatic equipment in a very brief time.

When these improvements are ready, Mrs. Housewife will be waiting for them and the new products we may expect will be at hand—due to the research teams which are pioneering on the food front to provide the new foods of the future.

THE ECONOMIC PICTURE

Before we turn to food research, let's take a brief look at the present economic status and importance of foods in the United States. In recent years:

The food industry is an 80 billion dollar industry. No other industry approaches this figure.

Over 21,000 of our supermarkets have sales totaling over five million dollars per year.

Foods now account for 27 per cent of the family spending in the United States.

In 20 years, the meat items have increased by a factor of nine; frozen foods by a factor of eight; baby foods by a factor of 42.

BUILT-IN CONVENIENCE

There are now 170 partly or fully cooked frozen products and 31 kinds of packaged cold meat cuts in the United States markets.

Today's food industry is a "package man's Utopia." Packaging competition is aimed at *impulse buying*. This raises the question of

the economics of food convenience which can be briefly illustrated by the following costs for foods for one day for four people:

Foods (prepared and almost ready for the table) requiring 90 minutes' preparation cost	$6.70
Foods requiring 180 minutes' preparation cost	5.80
Foods (requiring substantial preparation) requiring 300 minutes' preparation cost	4.70

Looking at it another way:

210 minutes (300 − 90) in the kitchen costs $2.00 ($6.70 − $4.70) at less than 60 cents per hour.

120 minutes (300 − 180) in the kitchen cost $1.10 ($5.80 − $4.70), or less than 60 cents per hour.

The kitchen has been taken to the factory. This explains the popularity of "Table-ready" foods to the housewife—if they are popular with the rest of the family.

Electronics and mechanics have made the kitchen a mechanical paradise with its dish washers, mixers, electronic ovens, grills, toasters, and openers. But appetite and discrimination remain, and quality will always be high on the totem pole!

Present statistical information indicates that with our increasing span of life we will have more "older citizens" because of better health, improved medical treatment, more hospitals, and more antibiotics. This means our food industries must provide more foods for persons with false teeth, crutches, and geriatric tastes, unless the population trends change.

HABITS, ATTITUDES, AND CONSUMPTION

Food habits are the basis for food consumption. Some habits change. The following foods which have recently become popular demonstrate the point: powdered coffee, baby foods, and pizza.

Changes in attitude also determine the fate of some products. Rancidity is no longer tolerated in any major United States food products—but it is not uncommon elsewhere! Changes in social habits also have their influence on the kinds of food we ingest. Cocktail accessories now encourage the expenditure of about 200 million dollars a year on potato chips—perhaps matched by the association of pretzels with beer. The advent of television has brought packaged dinners which require no dining room for the eating of complete precooked portable convenience dinners.

Diet and its attributes have brought their downward consumption trends in the potato industry and in the flour industry. Presently the fat and oil industries are beginning to note the reflection of a controversy which causes doubts in the minds of the consuming public.

Out-door eating and cooking will continue to alter the kinds of foods some persons eat, but in the northern states, because of cold winters, this influence will be more seasonal than elsewhere. What intelligent housewife objects to the fond husband doing his turn at the back-yard charcoal grill, using paper dishes which can be burned rather than washed, when the result is a family which has happily eaten the charred products he proudly produces?

Factors which affect food consumption and distribution are:

Convenience
- For example:
  - Packaged pie and cake mixes
  - Frozen bakery products
  - Canned or frozen specialties
  - TV dinners and finished desserts
  - Coin food machines and industrial feeding mechanizations
  - Foods in packages in which they can be cooked and served—no dishes to wash

Acceptability
- Foods must have satisfying:
  - Appearance
  - Package appeal
  - Flavor
  - Texture
  - Physical dimension

Efficiency and Economy
- Brought about by:
  - Good production practices
  - High speed production
  - Distribution cost reduction by supermarkets

Nutrition
- Today's foods have:
  - More built-in nutrition
  - Less processing damage
  - Recognized nutritional values
  - Broader variety usage
  - Greater nutritional stability

Safety and Sanitation
- Which are:
  - Expected in the United States
  - Usually provided by the processors and distributors
  - Largely insured by United States Food and Drug Administration and other regulatory agencies

THE SAFETY OF FOODS

The modern food industry also has brought *new legislation* to regulate what may and what may not be used in food production and food manufacturing, or what quantities of ingredients may be used. Food packages, too, must conform to highest standards of safety in respect to the ingredients they contain, which may transfer to the foods. Equipment and materials coming in contact with foods during handling and processing must also be such that they do not reduce the safety of the foods.

There is a most pressing need for information relating to functional components which may be in food products. We must know all that we can about the metabolic activities in food products in order to be sure they conform to safety and the law and so protect the consumer.

Additional scientific interest in this area is clearly needed. There is a dire need for more extensive facilities, definitely trained personnel (food toxicologists), and techniques to save time, effort and expense.

The ingenuity of food technology has resulted not only in new products, but also in new concepts of how to make them. For example: new continuous short-time breadmaking; new shortenings; new spreads; new instant milk; and new potato flakes.

NEW THINGS, OLD WAYS; OLD THINGS, NEW WAYS

The new concepts in food will always encompass the old standbys in food preservation, which include dehydration, canning by heat, freezing and frozen storage, and combinations of these. They may also include radiation by various wave lengths—high frequency, infra-red, electrons, and gamma rays. We may expect more foods preserved by antibiotics.

The wider use of improved dehydrated and concentrated beverages is assured and the acceptability of such products will constantly increase.

More fruits and vegetables will be kept at optimum acceptability for longer periods by controlled atmosphere gas storage: for example, apples. All kinds of shelf-fresh foods will be available at any time, summer or winter.

The recent institution of refrigerated bulk-tank holding of milk on the dairy farms combined with tank-truck pickup improves the quality of one of America's finest products—fresh milk.

We will see wider usage of enzymes in food products and in their preparation. More food products will be produced or supplemented through the use of micro-organisms and their synthetic abilities.

We will find many foods prepared especially for the aged, and products made particularly for hospitals and institutions. Individual servings or services of foods will increase tremendously as the number of individual housing facilities for the aged are provided, and this trend is going to rise in the near future. The "heat and eat" industrial and school "mechanized electronic feeding service" will supplant many cafeterias.

LOOKING FARTHER AHEAD

It is conceivable that we may have completely synthetic sugars and fats and proteins available. Rather than come from fields or orchards, barns or feed-lots, cane fields or beet acreages, they may come from laboratories which will put together, through the help of micro-organisms and enzymes, nitrogen from the air, carbon from flue-gases or petro-chemicals, and oxygen from equally simple and diverse origins. Science is on the march, the specialized and expensive techniques which today are making possible the synthesis of hormones, vitamins, antibiotics, and metabolic intermediates of various kinds in microgram or kilogram amounts will one day be utilized to turn out basic food materials by continuous process food engineering similar to that which has made our chemical industries capable of producing many other products.

But such possible changes and new things will not "just happen." Research is what creates the changes—the new products—the new business—and the obsolescence.

As this symposium has been keyed to enzymes, it will be apparent that many areas I have mentioned will need research in enzymology. The earlier speakers have indeed emphasized very clearly many of the factors which are an integral part of the vast picture. It is difficult, if not impossible, to imagine a food or a food process which does not involve either enzyme knowledge or an application of enzyme use or control in its success. Certainly this will be even more the case in

the future as we learn more of the unusual properties of these potentially very useful organic catalysts produced by living things.

The doors barely open to vast chambers of knowledge relating to enzymes—key to an infinite number of life-sustaining chain reactions. Basic knowledge will improve many foods, for the research of today becomes the application of food technology tomorrow.

A more complete understanding of the intricate enzyme mechanisms of the dynamic metabolic systems will result in:

More efficient conversions of animal feeds;
More efficient human food utilization;
More acceptable foods, with better flavor and flavor retention;
More effective methods of control for preventing deterioration; and
Possible activation of the sensory reactions relating to food acceptance, appetite, and food metabolism.

The thorough examination, exploration, and evaluation of the impacts of the enzymes could provide invaluable guidance to food manufacturers. If even some of these come to fruition great advances may be expected in food technology and food utilization.

To *Ernest H. Wiegand* and his followers who produce food products for Oregon, America and the world—

To those who follow in his footsteps and train food technologists—

To those who are dedicated to enzymes, their functions, characteristics, and utilization—

I express for the ordinary food consumers their appreciation for your successful efforts to date and best wishes for your continued success in the future for the ultimate benefit of all mankind.

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